

Bond University

DOCTORAL THESIS

Cytotoxic Drugs and their Effects on Bladder Function

Kang, Sung Hyun

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Cytotoxic drugs and their effects on bladder function

PhD Thesis

By

Sung Hyun Kang



Faculty of Health Sciences and Medicine

Bond University

Queensland, Australia

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Summary of Thesis

This thesis investigates the effects of intravesical agents, doxorubicin, mitomycin C (MMC), and epirubicin on release of mediators and inflammatory cytokines from the urothelium, and tissue responses and morphological integrity of the bladder. Intravesical therapy which administers cytotoxic agents into the bladder lumen is an important approach for treatment of superficial bladder cancer. Despite evidence of significant local adverse effects such as chemical cystitis (dysuria, increased urinary frequency and urgency) following this treatment, few studies have assessed their effects on non-malignant tissues of the bladder and the changes in urinary function resulting from this therapy. A better understanding of the mechanisms of bladder toxicity by these agents may lead to the identification of novel approaches for reducing the severity of reported adverse effects. Moreover, the effects of ageing on bladder function were also investigated in this thesis.

Two *in vitro* bladder models, human urothelial cell lines (RT4 & UROtsa) and porcine bladder tissues (young & aged) were used to evaluate the effects of the three cytotoxic drugs on bladder function at clinically relevant concentrations and durations of treatment. Tissue responses to carbachol, isoprenaline and electrical field stimulation (EFS) were assessed in isolated bladder tissues, and integrity of bladder structure was also evaluated in terms of changes in urothelial thickness. Urothelial mediators (ATP, ACh and PGE₂) were measured in both cells and tissues under basal and stretch-induced conditions (mechanically or hypo-osmotically). Release of inflammatory cytokines (IL-8, IL-1 β , IL-6, IL-10, TNF and IL-12p70) and nitric oxide (NO) were also measured, and recovery studies (24 hour- and 1 week-post treatment, and 1 week-post repeat treatment) were also conducted using urothelial cultures.

The first study comparing function of the bladder between young (4 to 6 months old) and aged (2 to 3 years old) pigs revealed age-associated decreases in contractile responses to muscarinic stimulation and also reduced urothelial thickness, while increases in urothelial mediator release, efferent neurogenic responses and relaxation responses of the urothelium/LP were observed with increasing age. In bladders from both young and aged pigs, pretreatment with doxorubicin enhanced ATP release from the urothelium/LP and also increased contractile responses of the

urothelium/LP to muscarinic stimulation. In bladders from young pigs, doxorubicin pretreatment enhanced efferent neurogenic responses of the detrusor muscle without affecting the muscle response itself, but in bladders from aged pigs, doxorubicin enhanced contractile response (muscarinic) of the detrusor muscle and also depressed neurogenic detrusor contractility.

The second study using bladders from aged pigs alone demonstrated that in tissues pretreated with MMC, efferent neurotransmission in detrusor muscle was depressed, relaxation of the detrusor muscle to adrenergic stimulation was reduced, and urothelial contractility to muscarinic stimulation was decreased, compared to control tissues. Pretreatment with both MMC and epirubicin caused urothelial thinning, abolished the ability of the urothelium to inhibit detrusor contractility, and depressed detrusor contractility to muscarinic stimulation. Also, stretch-induced release of ATP from the urothelium/LP was decreased after MMC and epirubicin pretreatment, while basal release of PGE₂ and ACh was increased by MMC and epirubicin, respectively.

Experiments using urothelial cells demonstrated a decrease in stretch-induced ATP release immediately and 1 week following MMC pretreatment, but recovery was observed 1 week after repeat MMC pretreatment. Basal release of ACh was enhanced immediately and 24 hours following pretreatment, but 1 week following pretreatment, basal ACh release was decreased while stretch-induced release of ACh was increased. Immediately following pretreatment, a decrease in basal PGE₂ release was observed. However, 24 hours and 1 week following pretreatment, an increase in basal PGE₂ release was demonstrated. One week after repeat pretreatment, stretch-induced release of PGE₂ was enhanced. In addition, enhancement in NO release 24 hours and 1 week following MMC pretreatment was shown, with recovery observed 1 week following repeat pretreatment.

Further experiments revealed that immediately and 24 hour following epirubicin pretreatment, both basal and stretch-induced ATP release were enhanced while stretch-induced ATP release was depressed 1 week following pretreatment. An increase in basal ACh release and a decrease in stretch-induced ACh release were observed immediately and 24 hour after pretreatment. One week following pretreatment, an increase in stretch-induced ACh release was observed. The release of PGE₂ was only affected immediately after pretreatment, in which basal release

was increased while stretch-induced release was depressed. NO release was enhanced 24 hour following pretreatment, but a recovery was observed 1 week after pretreatment.

One of the most important findings in this study was the persistent induction of inflammatory cytokines in urothelial cell culture models following pretreatment with the each of the chemotherapeutic drugs tested. All three agents enhanced IL-8 release, while IL-6 and IL-1 β were enhanced by epirubicin and doxorubicin, respectively. This suggests that changes in inflammatory response may be the key to pathogenesis of the urological adverse effects reported in bladder cancer patients treated with these intravesical agents.

Thus, these studies have identified a number of changes in bladder function that may contribute to the adverse effects observed following intravesical chemotherapy. A number of pathological changes were observed in muscle, nerve and urothelium/LP that may influence sensory and motor functions of the bladder, but the changes following treatment appear to differ for each drug and even the age of the bladder, the only consistent change being an inflammatory state that was observed with all the drugs tested.

Declaration of Originality

This thesis is submitted to Bond University in fulfilment of the requirements of the degree of Doctor of Philosophy. This thesis represents my own original work towards this research degree and contains no material that has previously been submitted for a degree or diploma at this university or any other institution, except where due acknowledgement is made.

Sung Hyun Kang

May 2015

Supervisor: Dr. Catherine McDermott. *Assistant Professor*, Faculty of Health Sciences and Medicine, Bond University.

Co-supervisor: Professor Russ Chess-Williams. *Professor of Pharmacology*, Faculty of Health Sciences and Medicine, Bond University.

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Chess-Williams, R., Kang, S. H., & McDermott, C. (2012). Is the release of urothelial ATP, acetylcholine and prostaglandin E2 affected by the chemotherapeutic agent doxorubicin? *Neurourol. Urodyn*, 31(6), 1020 (42nd Annual meeting of the International Continence Society, Beijing, China)

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Chapter 1 - Introduction

Bladder cancer

Epidemiology

Bladder cancer is the 9th most common type of cancer (429,800 new cases, 330,400 male and 99,400 females) and the 13th most common cause of cancer death (165,100 deaths, 123,100 male and 42,000 females) worldwide in 2012 (Ferlay et al., 2013). The incidence varies significantly between geographical regions and countries, being particularly high in many developed countries including southern and eastern European countries, parts of Africa and the Middle East, and North America (Parkin, 2008; Ploeg et al., 2009). The highest estimated mortality is in Egypt, where the rate was three times and eight times greater in comparison to Europe and the USA, respectively (Parkin, 2008). While, incidence rates of bladder cancer were declining or were stable in most Western countries in recent decades following a prior period of increase (American Cancer Society, 2015), relative survival for bladder cancer has decreased over time. Between the periods 1982-1987 and 2006-2010, five-year relative survival decreased from 67.9 per cent to 57.5 per cent in Australia while relative survival rate for most other cancers increased over the same time period (AIHW, 2012). Similar declining trends were also observed in England and Wales (Cancer Research UK, 2014). According to the Australian Institute of Health and Welfare, bladder cancer is the tenth most common cancer in Australia (AIHW, 2012). There were 2,459 new cases of bladder cancer diagnosed in Australia in 2010 and 1031 deaths occurred in 2011. Both incidence and mortality of bladder cancer is expected to grow higher (AIHW and AACR, 2012).

Age is a major risk factor for bladder cancer. It is rare under the age of 50 years but thereafter the risk increases with age. The overall median age at diagnosis is 70 years in men and women combined (Sexton et al., 2010). Gender is another important risk factor, with males having approximately three to four times higher risk of developing bladder cancer compared to women and the worldwide age standardised incidence rate is 10.1 per 100,000 for men and 2.5 per 100,000 for women (Ploeg et al., 2009). Its incidence is particularly high in industrialised countries mainly because of its association with tobacco use and exposure to industrial carcinogens. Evidence suggests that there are particular occupations that are at higher risk of developing bladder cancer than others. For example, people working in rubber, metal and mining industries as well as painters, leather workers and truck drivers have been identified

as having higher risks. Tobacco use is one of the most prominent risk factor for bladder cancer. Smokers have up to a 4-fold increased risk of developing bladder cancer compared with patients who have never smoked (Sexton et al., 2010).

Types and Histology of bladder cancer

The majority of bladder cancer patients (approximately 75-85%) initially present with a disease that is non-muscle invasive bladder cancer (NMIBC) of which 15% to 25% progress to muscle invasive bladder cancer or metastatic disease (Babjuk et al., 2011; Botteman et al., 2003). The most common type of bladder cancer is transitional cell carcinomas (TCC), accounting for approximately 90% of patients with bladder cancer (Avritscher et al., 2006; Sexton et al., 2010). Whereas 5% have squamous cell carcinomas and 1% to 2% have adenocarcinomas (Table 1.1) (Sexton et al., 2010).

Table 1.1: Bladder tumour histologies (Sexton et al., 2010).

Malignant
Transitional cell carcinoma (TCC)
Pure TCC
TCC with mixed features (squamous or glandular differentiation)
Micropapillary
Nested
Lymphoepithelioma-like
Squamous cell carcinoma
Adenocarcinoma (primary bladder)
Small cell carcinoma
Carcinosarcoma (mixed epithelial and mesenchymal elements)
Sarcomatoid (epithelial elements only)

Classification of bladder cancer

Classification of bladder cancer plays an important role in determining the appropriate treatment strategy and also predicting outcomes for patients. Tumour, Node, Metastasis classification (TNM) approved by the Union International Contre Le Cancer (UICC) has been widely accepted for classifying staging of bladder cancer. In general, the extent of tumour tissue invasion is defined from stage TX to stage T4 (Table 1.2). Non-muscle invasive bladder cancers which are confined to the first two layers of the bladder: epithelium and suburothelium (lamina propria) includes stage Ta, T1 and Tis. Muscle invasive bladder cancer is defined as tumours

that invade the detrusor smooth muscle or beyond (stage T2 to T4) (Botteman et al., 2003). There has been a change in the WHO grading of bladder cancer with the 2004 grading system incorporating a range of histologic descriptions such as Urothelial Carcinoma *in situ* (CIS), urothelial papilloma (completely benign lesion), and papillary urothelial neoplasm of low malignant potential (PUNLMP), rather than the previous three grades of well (Grade 1), moderately (Grade 2) or poorly differentiated (Grade 3) papillary urothelial carcinoma (Table 1.3).

Table 1.2: 2009 TNM classification of urinary bladder cancer (Babjuk et al., 2011)

T – Primary tumour	
Tx	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Ta	Non-invasive papillary carcinoma
Tis	Carcinoma in situ: ‘flat tumour’
T1	Tumour invades subepithelial connective tissue
T2	Tumour invade muscle
	T2a Tumour invades superficial muscle (inner half)
	T2b Tumour invades deep muscle (outer half)
T3	Tumour invades perivesical tissue
	T3a Microscopically
	T3b Macroscopically (extravesical mass)
T4	Tumour invades any of the following: prostate, uterus, vagina, pelvic wall, abdominal wall
	T4a Tumour invades prostate, uterus or vagina
	T4b Tumour invades pelvic wall or abdominal wall

Table 1.3: WHO grading in 1973 and in 2004 (Babjuk et al., 2011)

1973 WHO grading	
	Urothelial papilloma
Grade 1:	well differentiated
Grade 2:	moderately differentiated
Grade 3:	poorly differentiated
2004 WHO grading	
	Flat lesions
	Hyperplasia (flat lesion without atypia or papillary aspects)
	Reactive atypia (flat lesion with atypia)
	Atypia of unknown significance
	Urothelial dysplasia
	Urothelial Carcinoma <i>in situ</i> (CIS)
	Papillary lesions
	Urothelial papilloma (completely benign lesion)
	Papillary urothelial neoplasm of low malignant potential (PUNLMP)
	Low-grade papillary urothelial carcinoma
	High-grade papillary urothelial carcinoma

Symptoms and Diagnosis of bladder cancer

Painless haematuria is the single most common symptom presenting in patients with bladder cancer that alerts patients to seek medical advice. Additionally, irritative lower urinary tract symptoms such as urgency, increased urinary frequency and dysuria are also common but are more predominant in patients with CIS or invasive disease (Sexton et al., 2010).

For several decades, standard white light cystoscopy (WLC) has been used to detect and resect bladder tumours (Figure 1.1); however advances in technologies have improved the quality of cystoscopy and transurethral resection of bladder tumour (TURBT) (Cheung et al., 2013). Photodynamic diagnosis/blue-light cystoscopy (PDD/BLC) improves the detection rates for inconspicuous bladder cancer by instilling 5-aminolevulinic acid (5-ALA) dye or its hexyl ester hexaminolevulinate (HAL) into the bladder. The dye is absorbed by dysplastic tissue, enabling photosensitisation in which the abnormal tissue emits a red colour under blue reference light and normal tissue appears blue. PDD/BLC is recommended during initial TURBT to aid diagnosis, and in patients with positive urine cytology but negative WLC results (Mowatt et al., 2011; Witjes et al., 2010). Narrow-band imaging (NBI) cystoscopy without the use of dyes, improves the fine structure of bladder mucosal surface via deeper penetration enabled by the longer wavelength of light (Herr and Donat, 2008). The most widely used non-invasive test is urine cytology. While urine cytology has good specificity and sensitivity for the detection of high-grade tumours, it has not been adopted into routine standard of care, owing to poor sensitivity for low-grade tumours and expense (Cheung et al., 2013). Also, many urine biomarker tests for detecting bladder cancer have been developed, including Fluorescence *in situ* hybridisation (FISH) that detects urinary cells that have chromosomal abnormalities consistent with a diagnosis of bladder cancer (Junker et al., 2003), and nuclear mitotic apparatus protein (NMP) 22 which is a marker that can be detected in voided urine (Shariat et al., 2011).

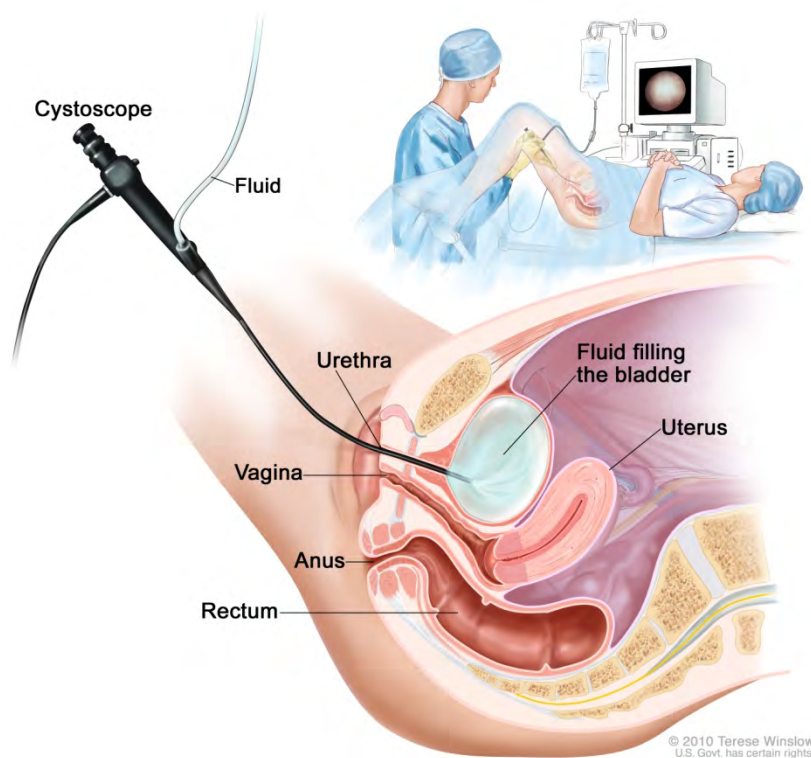


Figure 1.1: Illustration of how cystoscope is inserted through the urethra into the bladder to view the inner wall of the bladder on a computer monitor (Terese Winslow, 2010).

Treatment of bladder cancer

Transurethral resection of bladder tumour (TURBT)

For the initial management of non-muscle invasive bladder cancer, transurethral resection of bladder tumour (TURBT) is performed. Any visible tumours are resected using electrodes or laser during TURBT (Sexton et al., 2010). The information on the number of tumours, tumour size, tumour grade, the depth of invasion and prior recurrences collected during this procedure provide important data for risk stratification (Logan et al., 2012). Muscle-invasive bladder cancer requires more intensive therapy than non-muscle invasive bladder cancer. Generally, patients with muscle-invasive bladder cancer undergo a ‘radical cystectomy’, removal of the bladder and some surrounding organs such as uterus or prostate (Fry et al., 2010). Systemic chemotherapy where drugs are administered intravenously may be recommended as a treatment following a radical cystectomy (Hazard et al., 1952).

Intravesical treatment

Although TURBT can effectively remove a tumour completely, the high rate of recurrence and progression after TURBT necessitates the consideration of adjuvant treatments in all patients. The recurrence rates range from 48% to 70% and progression occurs between 7% and 40% of the time depending on tumour characteristics (Logan et al., 2012). Hence, after TURBT, patients receive treatment called an 'intravesical treatment' to reduce the re-implantation of tumour cells after TURBT, eradicate any residual disease, prevent tumour recurrence and reduce tumour progression. Intravesical treatment involves instillation of a therapeutic agent directly into the bladder via a catheter (Duque and Loughlin, 2000; Herr et al., 1995) (Figure 1.2).

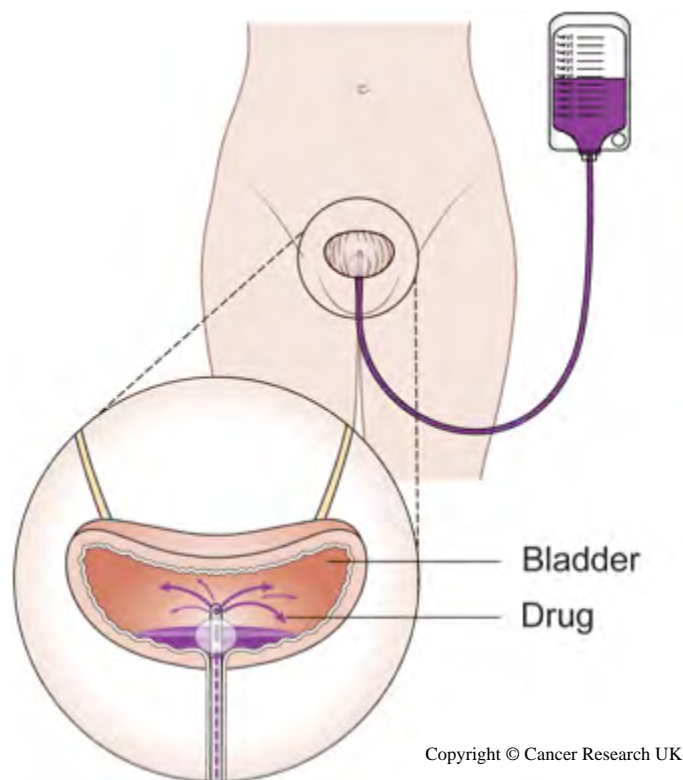


Figure 1.2: Diagram showing how intravesical agent is administered into the bladder (Cancer Research UK, 2013).

A study by Sylvester et al. (2004) found that one immediate postoperative instillation of chemotherapy after TURBT significantly reduced recurrence rate compared to TURBT alone from 48.4% to 36.7% (the reduction was 11.7%). Hence, guidelines published by the European Association of Urology (EAU) recommend one immediate postoperative dose of intravesical chemotherapy at the time or soon

(within 6 hours) after resection of suspected non-muscle invasive bladder cancer (Babjuk et al., 2011). However, an immediate postoperative instillation has to be avoided when there is overt or even suspicion of bladder wall perforation, since severe morbidity may occur if leakage outside the bladder into retroperitoneal or intraperitoneal space happens.

An additional adjuvant intravesical therapy is performed after TURBT. For at least six consecutive weeks, bladder cancer patients receive a weekly instillation of immunomodulatory or chemotherapeutic agents. Therapy is initiated approximately 2 to 4 weeks following the patient's TURBT and each instillation is performed for approximately 1 to 2 hours (Sexton et al., 2010). The most commonly used agents for intravesical chemotherapy and immunotherapy includes doxorubicin, epirubicin, mitomycin C (MMC) and Bacillus Calmette-Guerin (BCG).

Intravesical chemotherapy is an excellent targeted approach delivering effective concentrations of cytotoxic agents directly to the affected bladder lining. Administration of the drug through the urethra directly to the lumen of the bladder effectively minimizes systemic exposure to these toxic agents however there is evidence of local side effects (Dalton et al., 1991).

There is an extensive research effort focused on the enhancement of the toxicity of intravesical agents. Some examples include combination of intravesical chemotherapy with local hyperthermia or the use of nanocarriers (i.e. liposomes and solid lipid nanoparticles) for drug delivery (GuhaSarkar and Banerjee, 2010). However, there have been few studies that have assessed the effects of intravesical chemotherapeutic agents on normal bladder function. This is the focus of this thesis.

Intravesical immunotherapy

Intravesical use of the immunotherapeutic agent, Bacillus Calmette-Guerin (BCG) was first reported to be successful in treating superficial bladder cancer in 1976 by Morales et al. (1976). Intravesical BCG treatment is considered as the most successful immunotherapy to date (Chapman and Houghton, 1993; Ratliff et al., 1991) and is proven to be highly effective treatment for patients with intermediate- and high-risk non-muscle invasive bladder cancer (Babjuk et al., 2011; Brausi et al., 2011). An initial intravesical treatment with BCG for bladder cancer is

recommended by both the American Urological Association and European Association of Urology (Lightfoot et al., 2011).

The incidence of side effects is one of the main reasons why clinicians try to avoid the use of BCG. Intravesically administered BCG are generally well tolerated, however local and systemic adverse effects are reported to be more severe and more frequent compared with intravesical chemotherapy. During induction therapy, 5% of patients had to stop and 20% of patients stopped during maintenance therapy (van der Meijden et al., 2003). A study by Lamm et al. (1991) has reported that in patients with Ta and T1 tumours without carcinoma in situ, the probability of being disease free at 5 years was 17% after intravesical doxorubicin, as compared with 37 percent after intravesical BCG ($P<0.05$). The median times to treatment termination due to persistence (treatment failure), recurrence or progression of disease were 10.4 and 22.5 months, respectively. For patients with carcinoma in situ the estimated probability of documented disappearance of disease were 34% for doxorubicin (23 of 67 patients) and 70% for BCG (45 of 64 patients) ($P<0.001$). The median times to treatment failure were 5.1 and 39 months, respectively. The probability of being disease free at 5 years' survival was 18% and 45% respectively. Patients treated with BCG observed a higher incidence of toxic systemic effects and local irritative bladder symptoms than patients treated with doxorubicin.

The therapeutic mechanisms of BCG is under investigation; however available evidence suggests that an intact immune system, particularly the cellular system, is required for its antitumour activity (Prescott et al., 2000). Therefore, for elderly patients and in individuals who have impaired immune function, this agent is not an option (Heiner and Terris, 2008). Hence, chemotherapeutic agents such as doxorubicin, MMC and epirubicin are also commonly used for the treatment of bladder cancer as an alternative treatment option if immunotherapy fails or is ineffective (Lightfoot et al., 2011; Matsui et al., 2010).

Intravesical chemotherapy

Doxorubicin, mitomycin C (MMC) and epirubicin are the most widely used intravesical chemotherapeutic agents for the treatment of bladder cancer. Hence, they were used as representative chemotherapeutic agents for this research study where their effects on normal bladder function were assessed.

Doxorubicin

Doxorubicin (commercial name, Adriamycin) is a common chemotherapeutic drug for the treatment of bladder cancer and is classified as an anthracycline antibiotic (isolated from cultures of *Streptomyces peucetius* var. *caesius*) (Burden, 1998; Burden et al., 1998) which is composed of an aglycone and a sugar. The aglycone (doxorubicinone) consists of a tetracyclic ring with adjacent quinone-hydroquinone moieties and a short side chain with a carbonyl group (Salvatorelli et al., 2006). The sugar (daunosamine) is an amino-substituted trideoxy fucosyl moiety (Figure 1.3). Doxorubicin is also used systemically in the treatment of other types of cancer including acute lymphoblastic leukemia, acute myeloblastic leukemia, Wilms' tumor, neuroblastoma, soft tissue and bone sarcomas, breast carcinoma, ovarian carcinoma, transitional cell bladder carcinoma, thyroid carcinoma, gastric carcinoma, Hodgkin's disease, malignant lymphoma, and bronchogenic carcinoma (Fornari et al., 1994).

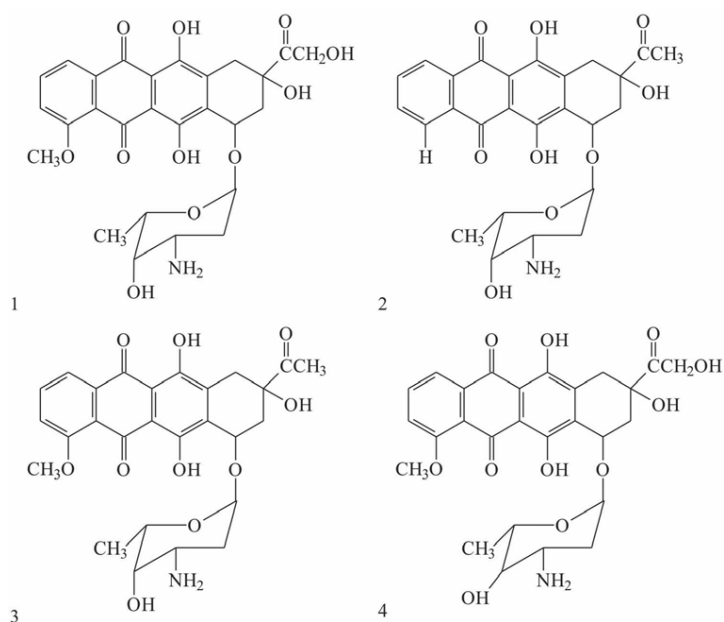


Figure 1.3: Structures of anthracyclines in current clinical use: (1) doxorubicin, (2) idarubicin, (3) daunorubicin, and (4) epirubicin (Cutts et al., 2005).

The most commonly used clinical dose of intravesical doxorubicin is 50mg in 50mL of saline retained in the bladder for 1 hour (Cheng et al., 2005; Cheng et al., 2004; Ilett et al., 1990).

Due to the high molecular weight of doxorubicin (580kd), systemic side effects are rare but the major systemic adverse effects which may limit its use is cardiotoxicity. The incidence of acute cardiotoxicity is approximately 11%, usually observed as chest pain due to myopericarditis and/or palpitations (Swain et al., 2003; Takemura and Fujiwara, 2007). The incidence of chronic doxorubicin cardiotoxicity (cardiomyopathy and arrhythmias) is much lower with estimated incidence of about 1.7% (Von Hoff et al., 1979). Systemic use of doxorubicin is also reported to be causing myelosuppression in some patients (Bally et al., 1990). Local side effects are relatively common in patients following intravesical treatment. Chemical cystitis presenting with symptoms such as dysuria (painful urination), increased urinary frequency and also hematuria (blood in the urine) are the most common side effects of doxorubicin (Badalament and Farah, 1997; Duque and Loughlin, 2000). An *in vivo* study using mice by Post et al. (1995) has shown a transient 3-fold increase in the urinary frequency induced by doxorubicin. Furthermore, intravesically administered doxorubicin has been reported to cause bladder toxicity in 13 to 56% of patients (Thrasher and Crawford, 1992). In a report by Ausfeld et al. (1987), 6.6% of patients treated with doxorubicin stopped the treatment due to toxicity.

The mechanism of action for doxorubicin and other anthracyclines have been extensively studied during the last several decades, but the mechanism still remains unclear and controversial (Yang et al., 2014). One of the potential mechanisms by which doxorubicin exert its cytotoxic effects on cancer cells is its action as topoisomerase II poison which targets topoisomerase II enzyme to induce apoptosis of cells. In addition, intercalation of doxorubicin and the formation of DNA adducts at active promoters, which increase stress and enhance nucleosome turnover, is another potential mechanism of doxorubicin-mediated cell death. Furthermore, doxorubicin also results in the generation of reactive oxygen species (ROS) which can damage DNA and cause cell death (Figure 1.4) (Yang et al., 2014).

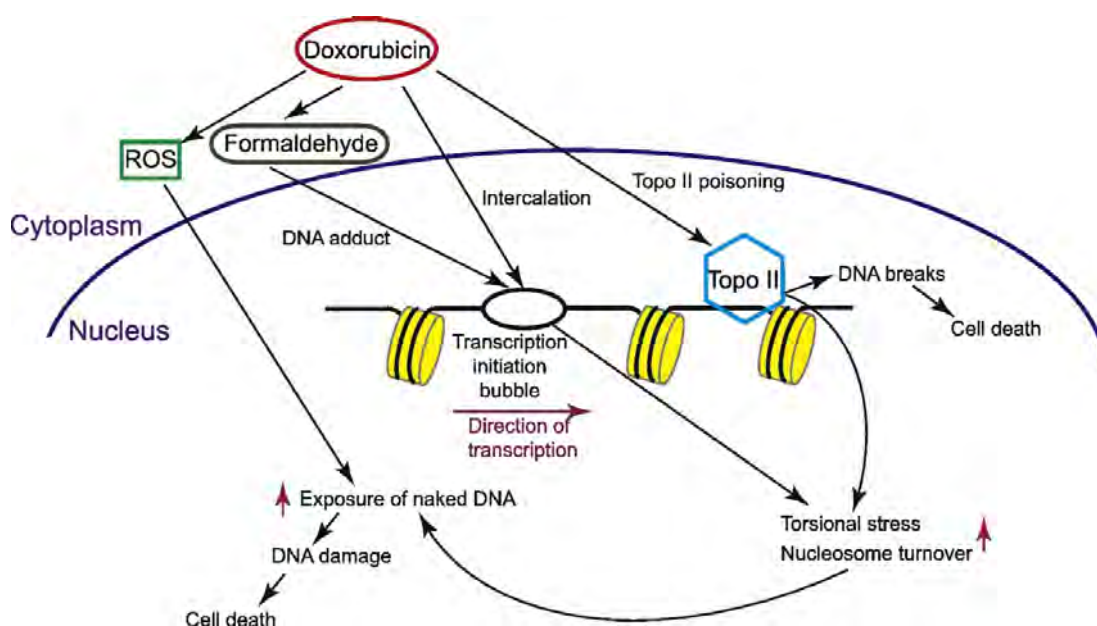


Figure 1.4: Graphical illustration of the potential mechanisms of doxorubicin-mediated cell death which includes poisoning of topoisomerase II enzyme, generation of reactive oxygen species, intercalation of doxorubicin and the formation of DNA adducts (Yang et al., 2014).

Doxorubicin along with other anthracyclines is one of the topoisomerase II poisoning agents (Nitiss, 2009). Topoisomerases are found in virtually all life forms, from bacteria to humans, and they are highly conserved enzymes that regulate DNA topology to facilitate DNA replication, transcription, and other nuclear process. Topoisomerase II is an ATP-dependent enzyme and there are two isoforms found in humans, topoisomerase II α and topoisomerase II β (Pommier et al., 2010). The enzyme binds DNA supercoils and entangled DNA, breaks both strands of one DNA duplex (called the ‘gate’ or G segment), passes the other DNA duplex (called the T segment) through this transient cleavage and reseals the break. This process results in the release of torsional stress formed during biological processes such as DNA replication and transcription (Liu et al., 1983; Pommier et al., 2010; Tewey et al., 1984; Wang, 1996). Doxorubicin interferes with enzymatic DNA breakage-reunion by trapping topoisomerase II on DNA in a covalently bound state (Capranico and Zunino, 1992; Liu, 1989; Tewey et al., 1984). The complex composed of enzyme, drug, and DNA has been termed the ‘cleavable complex’ because its disruption with detergent results in DNA double strand-break and covalent attachment of each subunit in the topoisomerase II homodimer, one to each 5’ phosphate end via a phosphotyrosyl linkage (Capranico and Zunino, 1992; Deffie et al., 1989; Liu, 1989). This stabilizes the cleavage complex and impedes religation of cleaved duplex, a

lesion that results in a DNA double-strand break. An apoptotic response occurs when these DNA double-strand break are not repaired (Liu et al., 1983; Tewey et al., 1984; Wu et al., 2011). Thus, the interaction of doxorubicin with topoisomerase II to form DNA-cleavable complexes appears to be an important mechanism of doxorubicin cytotoxic activity (Fornari et al., 1994; Momparler et al., 1976). In addition, decatenation of DNA during mitosis is topoisomerase II specific reaction, therefore doxorubicin which poisons topoisomerase II prevents cytokinesis resulting in cell death (Carpenter and Porter, 2004). The cytotoxic activity of doxorubicin on cancer cells is most likely to be due to its effects on dividing cells by topoisomerase II α whereas, cytotoxic effect of doxorubicin on normal cells which causes heart muscle failure is a side effect resulting from damage to non-dividing cells by topoisomerase II β . It has been shown that cardiomyocyte-specific deletion of topoisomerase II β protected mice from developing heart failure induced by doxorubicin (Zhang et al., 2012a). Also, topoisomerase II inhibitors have been shown to protect cardiomyocytes from doxorubicin-induced toxicity (Vavrova et al., 2013). These findings suggest that trapping topoisomerase II by doxorubicin especially topoisomerase II β in non-dividing heart cells may be responsible for doxorubicin-induced cardiotoxicity (Yang et al., 2014).

Doxorubicin-DNA adducts were originally characterised in a cell-free activation system, where transcriptional blockages induced by doxorubicin were observed at 5' GpC sequences (Cullinane and Phillips, 1990), demonstrating that doxorubicin formed covalent adducts with DNA at these sites (Swift et al., 2006). Doxorubicin which is a DNA intercalator prefers the intercalation site containing adjacent GC base pairs. This may be due to formation of specific hydrogen bond between doxorubicin and guanine (Chaires et al., 1987; Chaires et al., 1990; Chen et al., 1986). Doxorubicin-DNA adducts have been shown to activate DNA damage responses and induce cell death that are independent of topoisomerase II (Forrest et al., 2012; Swift et al., 2006). Further analysis of the cell-free activation system used to form adducts has shown that formaldehyde was a byproduct of the reaction conditions (Taatzjes et al., 1997). Doxorubicin-DNA adducts can be stabilised by a covalent bond mediated by cellular formaldehyde that is generated by free radical reactions from carbon sources such as lipids and spermine (Taatzjes et al., 1996, 1997). This interaction involves formation of a covalent bond between 3' amino group of doxorubicin and the N2 of guanine on one strand of DNA mediated by formaldehyde and of a

hydrogen bond between doxorubicin and guanine on the opposing strand of DNA (Figure 1.5) (Kato et al., 2000; Kato et al., 2001; Taatjes et al., 1997). A study by Coldwell and his colleagues (2008) has demonstrated that doxorubicin-DNA adducts are detectable at clinically relevant drug concentrations. Despite the evidence of DNA adducts formation during doxorubicin treatment, the major mechanism of cytotoxic action of doxorubicin is unlikely to be due to formation of DNA adducts since clinical doses result in only 4.4 ± 1.0 adducts/ 10^7 base pair DNA, which accounts for just a small fraction of total doxorubicin (Coldwell et al., 2008). Furthermore, it has been shown that preactivated form of doxorubicin, doxoform can be produced by formaldehyde which consists of two doxorubicin molecules bound together with three methylene groups (Fenick et al., 1997). Doxoform is approximately 200-fold more cytotoxic than doxorubicin alone, displays an accelerated uptake by cells, is retained longer in nucleus, and is particularly cytotoxic to doxorubicin-resistant cell line (Fenick et al., 1997; Taatjes et al., 1999).

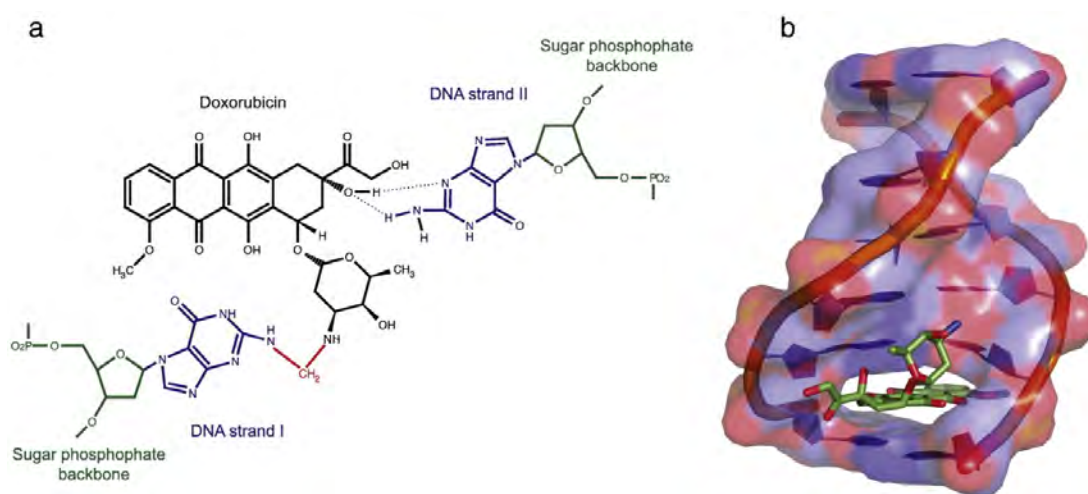


Figure 1.5: Structure of the doxorubicin-DNA complex. (a) Doxorubicin forms a covalent bond (shown in red) with guanine on one strand of DNA mediated by formaldehyde and hydrogen bonds with guanine on the opposing strand. (b) A structure of intercalation of doxorubicin into DNA. Doxorubicin intercalates into DNA and pushes apart the flanking base pairs with the sugar moiety sitting in the minor groove (Yang et al., 2014).

Doxorubicin-mediated generation of reactive oxygen species (ROS) has been discussed as one of the possible mechanisms of cytotoxic effects of doxorubicin on both cancer cells and normal cells (for example doxorubicin causes cardiotoxicity) (Figure 1.4). Several mechanisms by which doxorubicin induce generation of ROS

have been proposed. The quinone structure of doxorubicin is prone to the generation of ROS through enzymatic mechanism utilizing mitochondrial respiratory chain and non-enzymatic pathway, which incorporates iron (Gutierrez, 2000; Shadle et al., 2000). The quinone moiety of doxorubicin can be oxidised to a semiquinone radical through one-electron reduction mediated by several enzymes including cytochrome P450 reductase, NADPH dehydrogenase, endothelial nitric oxide (NO) synthase and xanthine oxidase (Berlin and Haseltine, 1981; Childs et al., 2002; Davies and Doroshov, 1986; Fogli et al., 2004; Kostrzewa-Nowak et al., 2005; Menna et al., 2007; Minotti et al., 2004; Pawlowska et al., 2003; Ravi and Das, 2004). Once semiquinone radical has been generated, it can quickly react with oxygen to generate reactive oxygen species including superoxide and hydrogen peroxide to causing DNA damage (Berlin and Haseltine, 1981; Minotti et al., 2004) or be oxidised back to the quinone form (Ramji et al., 2003). Hence, release of free radicals induced by doxorubicin may cause oxidative stress, resulting in DNA damage and cell death (Thorn et al., 2011).

It has been postulated that doxorubicin may cause cardiotoxicity via both depression of cardiac antioxidant levels, particularly glutathione peroxidase, and increased generation of oxygen free radical, thereby overwhelming the limited cardiac defence system (Doroshov and Davies, 1983). However, a study by Alderton et al. (1992) has demonstrated that neither doxorubicin nor doxorubicin in conjunction with cardioprotective agent (ICRF-187) caused any significant effect on the cardiac antioxidant capacity of mice. Thus, it appears that doxorubicin cause cardiac damage via the generation of oxygen free radicals rather than depression of antioxidant defences. In addition, doxorubicin which is an iron chelator can strongly bind iron through its quinone-hydroquinone functional groups and this complex catalyses the conversion of hydrogen peroxide to highly reactive hydroxyl radicals (Myers, 1998; Swain et al., 1997). Furthermore, doxorubicin has been reported to be inducing generation of ROS via alteration of Ca^{2+} homeostasis (Kim et al., 2006c). It has been demonstrated that doxorubicin-mediated ROS generation and apoptosis are inhibited by reducing intracellular Ca^{2+} levels using Ca^{2+} chelator (Kalivendi et al., 2005; Kalivendi et al., 2001). Also, doxorubicin has been reported to increase the open probability of sarcoplasmic reticulum (SR) calcium release channels (Kim et al., 1989; Zorzato et al., 1985), inhibits $\text{Na}^{+}\text{-Ca}^{2+}$ exchanger (Caroni et al., 1981), or activates L-type cardiac calcium channel (Keung et al., 1991). These effects of

doxorubicin on Ca^{2+} channels can result in Ca^{2+} overload of cardiac cells that may lead to mitochondrial calcium overloading, causing alteration of energy metabolism and generation of ROS (Kim et al., 2006c). To protect patients treated with doxorubicin from cardiotoxicity, free radical scavengers have been used but many of them failed. This suggest that free radical formation is not the only mechanism of cardiotoxicity (De Beer et al., 2001).

Epirubicin

Epirubicin (commercial name, Ellence) is another anthracycline drug which is a modern derivative (epimer) of doxorubicin. Epirubicin differs from doxorubicin in an axial-to-equatorial epimerisation of the hydroxyl group at C-4' in daunosamine (the sugar) (Figure 1.6) (Salvatorelli et al., 2006). This drug is favoured over doxorubicin, the most popular anthracyclines, in some chemotherapy regimens because it has an equivalent spectrum of antitumor action to that of doxorubicin but exhibits less systemic and cardiac toxicity (El-Mahdy Sayed Othman, 2000; Perreault et al., 2013; Smith et al., 2010).

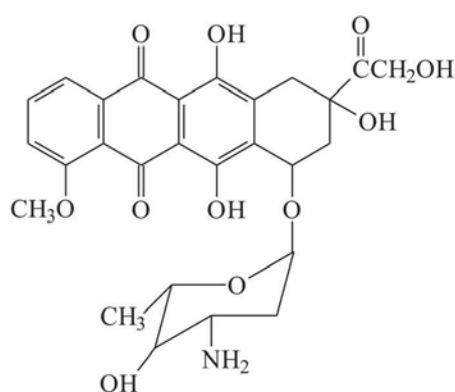


Figure 1.6: The chemical structure of epirubicin (Cutts et al., 2005).

It is commonly used as an intravesical chemotherapeutic agent for the treatment of bladder cancer (Onrust et al., 1999) and is also used to treat various types of cancer, including breast cancer, ovarian cancer, stomach cancer, lung cancer, bowel cancer, myeloma and some types of lymphoma and leukaemia (Cersosimo and Hong, 1986; Coukell and Faulds, 1997; Muggia and Green, 1991; Vermorken et al., 1999). After intravenous administration to patients, depending on dose, epirubicin causes bone marrow depression (decrease in erythrocyte, leukocyte and thrombocyte in blood) as

well as nausea, vomiting, diarrhea, lack of appetite and cardiac toxicity (Ulakoglu and Altun, 2004).

The common dosage of intravesical epirubicin is 50mg in 50mL of saline retained in the bladder for 1 hour. The dose can be increased to 80mg in patients with carcinoma *in situ* (Onrust et al., 1999). The concentrations of epirubicin and durations of treatment tested in the studies described in this thesis are based on these clinical values.

Major adverse effects associated with intravesical epirubicin were localised to the bladder which included chemical cystitis, urinary tract infection and haematuria. In addition, contracted bladder and haemorrhagic cystitis occurred in a few trials. Systemic adverse events after intravesical epirubicin were not reported in many trials (cardiac, haematological or related to hypersensitivity) (Hendricksen et al., 2008; Onrust et al., 1999).

The exact mechanisms of antitumor action of epirubicin have not been completely elucidated. Cytotoxic activity of epirubicin is active in all phases of the cell cycle and it is most active in the S and G₂ phases (Coukell and Faulds, 1997; Ozcan et al., 1997). It was demonstrated that epirubicin extended the duration of meiotic divisions in mouse spermatocytes for 48 hour (Attia et al., 2014). Therefore, this observation supports previous findings that inhibition of topoisomerase II function induced by epirubicin during different phases of the cell cycle slows down cell cycle progression and causes cells to arrest at the G₂/M phase (Coukell and Faulds, 1997). Epirubicin like its parent drug, doxorubicin is also a topoisomerase II poison. Topoisomerase II normally functions during the process of DNA replication to produce transient double strand breaks in the replicating DNA to relieve torsional stress, and reseals the break (Liu et al., 1983; Pommier et al., 2010; Tewey et al., 1984; Wang, 1996). Epirubicin interferes with this enzymatic DNA breakage-reunion by trapping topoisomerase II on DNA in a covalently bound state to form the drug-DNA-topoisomerase II ternary complex, which is called the cleavable complex. Thus, epirubicin inhibits the resealing of DNA breaks created by topoisomerase II, resulting in cytotoxic activity (Cantoni et al., 1989; Niitsu et al., 2000; Plosker and Faulds, 1993; Spadari et al., 1986).

The other mechanism of epirubicin's cytotoxicity is intercalation of epirubicin between DNA base pairs which leads to the inhibition of DNA replication and transcription (Lollini et al., 1989; Piestrzeniewicz et al., 2004; Skladanowski and Konopa, 1994; Wilmanska et al., 2001). Epirubicin also interferes with DNA unwinding or DNA strand separation and helicase activity. In addition, epirubicin induces generation of free radicals leading to DNA damage or lipid peroxidation (which results in cell damage) (Cantoni et al., 1989; Minotti et al., 2004). Also, a study by Malisza and Hasinoff (1995) demonstrated that epirubicin, bound to Fe (III), can produce highly reactive hydroxyl radical by enzymatic and non-enzymatic mechanism.

Epirubicin was found to be less toxic than doxorubicin in animal models (Alderton et al., 1992; Llesuy et al., 1990; Robert, 2007) and also less cardiotoxicity for epirubicin was demonstrated in some *in vivo* studies (Dardir et al., 1989; Weiss, 1992). The dose of epirubicin equicardiotoxic to 1mg of doxorubicin has been reported to be 1.8mg for cardiac symptoms and 2.2mg for injury on endomyocardial biopsies (Henderson et al., 1982). However, meta-analysis of randomised clinical trial data conducted by van Dalen and his colleagues (2010) concluded that there was no significant difference between occurrence of clinical heart failure between doxorubicin and epirubicin. The exact reason why epirubicin is less cardiotoxic than doxorubicin is unknown but it may be associated with its ability to yield oxygen free radicals and/or its stimulation of lipid peroxidation. Studies using acute and chronic models which measured endogenous lipid peroxidation and hydroperoxide-initiated chemiluminescence demonstrated that the hearts exhibited less oxidative stress for epirubicin compared with doxorubicin (Llesuy et al., 1990; Llesuy et al., 1985). However, other investigations have been unable to find any difference in the ability to generate superoxide anions (Grankvist and Henriksson, 1987) or promote lipid peroxidation (Vile and Winterbourn, 1989) between the two anthracyclines. A study by Alderton et al. (1992) has demonstrated that neither epirubicin nor epirubicin in conjunction with cardioprotective agent (ICRF-187) caused any significant effect on the cardiac antioxidant capacity of mice. Therefore, it is likely that epirubicin causes cardiac damage by the generation of oxygen free radical rather than compromised antioxidant defences.

Alternatively, it has been discussed in the previous section that doxorubicin may be cardiotoxic by altering cardiac calcium concentrations (Kim et al., 2006c). Epirubicin causes less inhibition of calcium turnover in cultured heart cells (Villani et al., 1980) and less inhibition of $\text{Na}^+ - \text{Ca}^{2+}$ pump (Caroni et al., 1981) compared to doxorubicin, which may explain its lower cardiotoxicity. Another contributing factor for lower cardiotoxicity of epirubicin may be the fact that it has different pharmacokinetic properties compared to doxorubicin. The minor structural difference compared to doxorubicin makes epirubicin a much better substrate than doxorubicin for human liver UDP-glucuronosyltransferase-2B7 (UGT2B7) (Dardir et al., 1989). Unlike doxorubicin, epirubicin undergoes glucuronidation (Torti et al., 1986). Glucuronidation, which transfers a highly hydrophilic glucuronide group to hydrophobic substrates, is a major detoxification pathway for numerous endo- and xenobiotics. The resulting glucuronide products are generally more easily excreted in bile and urine and less toxic than the initial molecules (Dardir et al., 1989).

Mitomycin C

The mitomycins are a group of highly potent antibiotics first isolated in 1950's in Japan from *Streptomyces caespitosus* (Hata et al., 1956). Currently 17 mitomycins are known and sixteen of the known mitomycins have shown broad spectrum antibiotic and potent antitumor activity. One particular member of the family, mitomycin C (MMC) (commercial name, Mutamycin/Ametycine), has been used clinically for cancer chemotherapy since the 1960's. It is currently one of the most important cytotoxic drugs in the treatment of cancer due to having a wide clinical antitumour spectrum with efficacy in various tumour types including gastric cancer, pancreatic cancer, breast cancer, non-small cell lung cancer, cervical cancer, prostate cancer and bladder cancer (Rjiba-Touati et al., 2014; Verweij and Pinedo, 1990).

The most commonly used clinical dose of intravesical MMC is 40mg in 40mL of sterile water administered for 1 to 2 hours. Higher concentrations may show additional benefit (Logan et al., 2012). A study by Au et al. (2001) showed that higher concentration of MMC (40mg in 20mL) improved its efficacy with only dysuria occurring more frequently compared to the patients treated with normal dose of MMC (20mg/20mL), but did not lead to more frequent treatment termination.

Owing to the high molecular weight (334 kDa) of MMC, systemic absorption is low and hence, systemic reactions are uncommon (Pashos et al., 2002). It has been reported that only 3% of intravesically administered MMC reaches the urothelium (Wientjes et al., 1993). However, common adverse side effects associated with the systemic use of MMC include bone marrow toxicity (thrombocytopenia and leukocytopenia), with cardiotoxicity (congestive heart failure), nephrotoxicity (haemolytic uremic syndrome), pulmonary toxicity (pneumonitis) and hepatotoxicity occurring less frequently (Rjiba-Touati et al., 2014; Verweij and Pinedo, 1990). The adverse effects associated with intravesical MMC are confined to local site.

Chemical cystitis has been reported in 41% of patients which includes dysuria, increased urinary urgency and frequency, suprapubic pain and discomfort (Kamat and Lamm, 2000). In addition, decreased bladder volume and a contracted bladder have been reported in patients (Duque and Loughlin, 2000; Kim and Lee, 1989). These cases are consistent with the finding by Michielsen et al. (2005) which observed decreased volume and compliance of the bladder in mice treated with MMC. Three weeks after the cessation of therapy recovery of bladder function was not observed in MMC treated mice (Michielsen et al., 2005). There have also been reports of allergic skin reactions and myelosuppression, which is rare (Koya et al., 2006; Sylvester et al., 2002).

MMC itself as a prodrug does not have significant cytotoxicity. However, upon enzymatic or chemical reductive activation, highly reactive monofunctional and bifunctional DNA-alkylating agents (six covalent DNA adducts) are formed which can cause lethal intrastrand and interstrand DNA cross-links (Bargonetti et al., 2010; Tomasz and Palom, 1997). The intracellular activation of MMC is catalysed by endogenous flavoreductases and proceeds by either anaerobic one electron reduction or oxygen-independent two-electron reduction. Various enzymes that are capable of activating MMC have been characterised within the cells. These includes NADPH dehydrogenase, quinone 1(NQO1; DT-diaphorase) (Siegel et al., 1992; Siegel et al., 1990) and xanthine dehydrogenase (Gustafson and Pritsos, 1992) which are able to activate MMC by a two-electron reduction. Also, NADPH-cytochrome P450 reductase, xanthine oxidase (Pan et al., 1984) and cytochrome *b*₅ reductase (Hodnick and Sartorelli, 1993) are responsible for one-electron reduction. Degree of toxicity

can vary depending on whether MMC undergoes one- or two-electron reduction (Snodgrass et al., 2010).

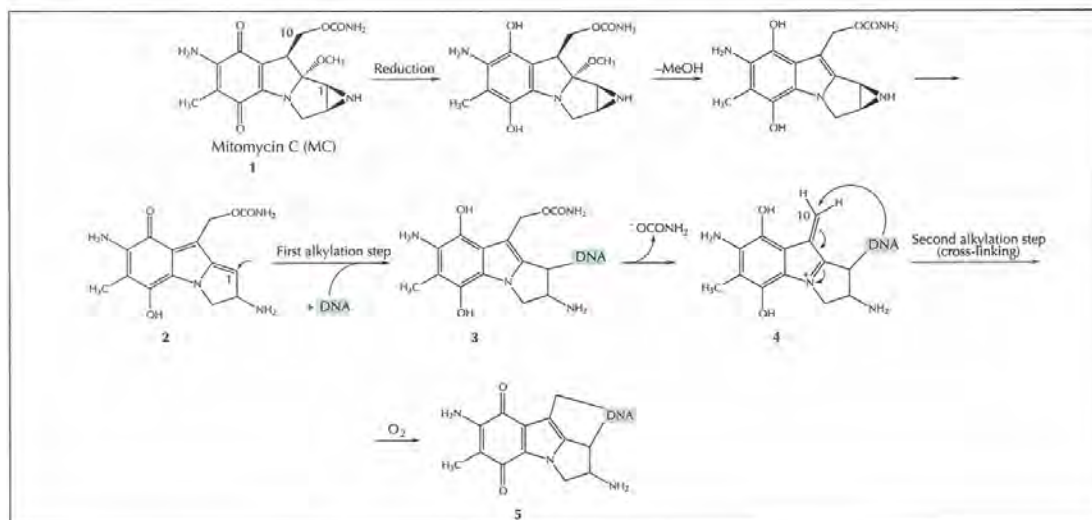


Figure 1.7: The reductive activation of mitomycin C (MMC). Reduction of the quinone of MMC initiates a cascade of spontaneous transformations that results in the production of quinone methide (2). When compound 2 reacts with a DNA nucleophile at C1 (to form adduct 3), a second alkylating center develops at C10 by reverse Michael elimination of the carbamate (adduct 4). Adduct 4 is attacked by another DNA nucleophile to give the reoxidised DNA crosslink (adduct 5) (Tomasz, 1995).

A key step in both routes is the enzymatic reduction of the quinone moiety of MMC, which initiates a cascade of spontaneous transformations that result in the production of quinone methide (2) which has high alkylating reactivity at the C1 position (Cummings et al., 1998; Spanswick et al., 1998). Adduct 3 is formed when compound 2 reacts with a DNA nucleophile at C1. During this process, second alkylating center develops at C10 by reverse Michael elimination of the carbamate and forms adduct 4. Adduct 4 is attacked by another DNA nucleophile to give the reoxidised DNA crosslink (adduct 5) (Figure 1.7) (Moore, 1977; Szybalski and Iyer, 1964; Tomasz, 1995; Tomasz and Lipman, 1981; Tomasz et al., 1987). The fact that MMC is activated by reduction plays significant role in the selectivity of its antitumour activity. Many solid tumours compared to normal tissues are in shortage of oxygen. As activation of MMC is inhibited by an oxidizing environment, toxicity of MMC is selective for these hypoxic solid tumours, and potently inhibits their growth (Sartorelli et al., 1994).

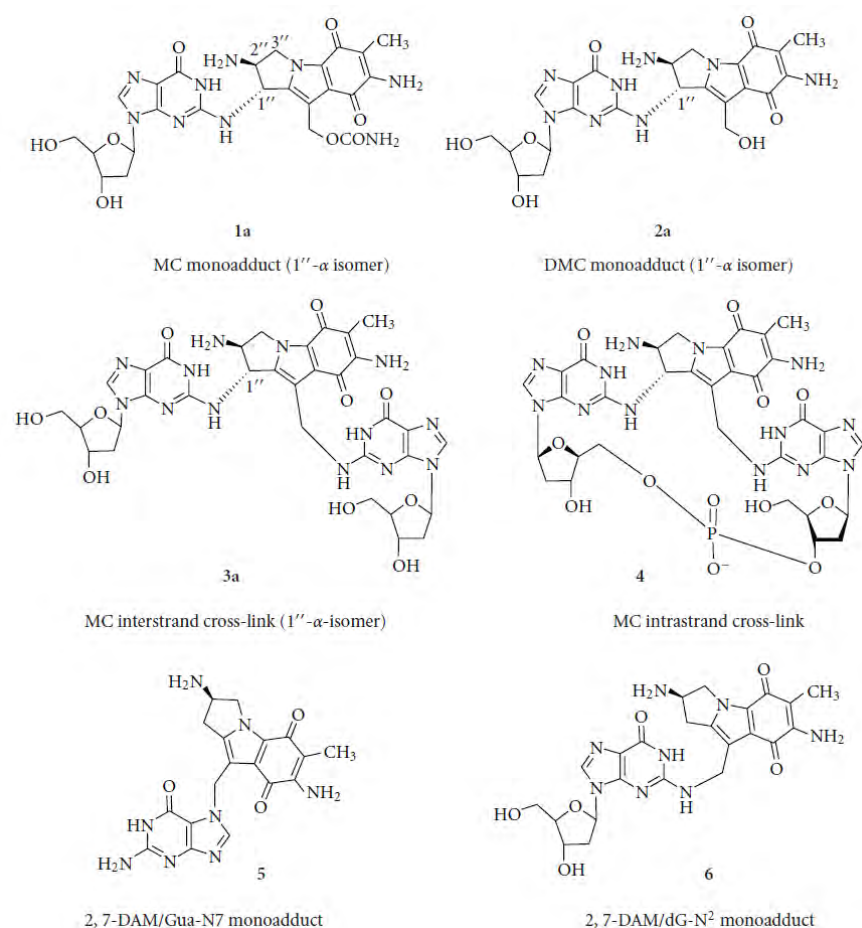


Figure 1.8: Chemical structure of six major adducts of reductively activated MMC (Bargonetti et al., 2010).

The structures of six major adducts of reductively activated MMC are shown in Figure 1.8. The first alkylation step by the quinone methide leads to MMC monoadducts (1''-α isomer) (**1a**). In some conditions, this is the only alkylation that occurs (Tomasz et al., 1986). Under conditions in which the second alkylating center develops, MMC biadducts (1''-α isomer) (**3a**) is a major product which forms a crosslink between the DNA strands (Tomasz et al., 1987). At much lower frequency, the second alkylation step also produces another MMC biadduct (**4**) which forms a crosslink with one strand (Bizanek et al., 1992). Decarbamoyl mitomycin C (DMC) monoadduct (1''-α isomer) (**2a**) is also a major product of monofunctional activation, and results from nucleophilic attack by water at C10'' position (Tomasz et al., 1988). The same adducts have been detected and identified *in vivo* (Bizanek et al., 1993; Tomasz et al., 1987). In addition, monofunctional activation of DNA by the metabolite of MMC, 2,7-diaminomitomycin C (2,7-DAM) forms two adducts, 2,7-DAM/Gua-N7 (**5**) and 2,7-DAM/dG-N² (**6**) (Palom et al., 1998; Palom et al., 2000).

It has been reported that 2,7-DAM is not cytotoxic and does not activate the p53 pathway (Abbas et al., 2002). Conversely, MMC and DMC are cytotoxic and able to activate the p53 pathway (Abbas et al., 2002; Boamah et al., 2007). DMC appears to be more cytotoxic than MC and can also kill p53-deficient cells (Abbas et al., 2002; Boamah et al., 2007) by inducing degradation of Checkpoint 1 protein, which is not seen with MMC treatment of the p53-deficient cells (Boamah et al., 2007).

The first, monoalkylation step which forms crosslinked adduct is selective for guanine nucleosides in the sequence 5'-CpG-3' (Kumar et al., 1992; Li and Kohn, 1991) but not dramatically. Alkylation at CpG is on average 5-fold (Kohn et al., 1992) or 10-fold (Kumar et al., 1992) enhanced over that at other NpG sequences. In contrast, the second step is absolutely selective for a G opposite the C. G residues flanking the NpG sequence on the opposite strand are never crosslinked, though intrastrand crosslinks can form on NpGpG sequence (Borowy-Borowski et al., 1990; Millard et al., 1990; Teng et al., 1989).

Despite of this direct evidence for MMC-DNA adduct from cell-free or *in vitro* studies, there is evidence suggesting that DNA may not be the primary target of the mitomycin C *in vivo*. Snodgrass and his colleagues (2010) proposed that MMC-rRNA interactions are more functionally plausible with respect to drug distribution and metabolism than the current hypothesis focusing on MMC-DNA interactions. Since most enzymes that reduce MMC are located in the cytosol, it suggests that the drug activation *in vivo* is also cytosolic (Snodgrass et al., 2010). Due to the chemical similarities to DNA and the relative abundance of RNA in the cytosol, RNA appears to be a potential primary cellular site for MMC activation. Also, the fact that rRNA constitutes approximately 71% of total cellular RNA in eukaryotes and as well as the fact that it contains G/C rich regions for preferred MMC binding (Schmid et al., 1994) suggest that rRNA is likely the primary cellular RNA target. It has been speculated that MMC rapidly binds 18S rRNA in the cytosol causing degradation and decreases levels in the cytosol. Decreases of this magnitude can potentially prevent the formation and/or function of ribosomal complexes inducing cell death through genome-wide translational silencing (Snodgrass et al., 2010).

Economic burden

Overall, bladder cancer has the highest lifetime treatment costs per patient of all cancer (Sievert et al., 2009) and is the 5th most expensive cancer in terms of total medical care expenditures, accounting for almost \$US3.7 billion (2001 values) in direct treatment-related costs in the US (Botteman et al., 2003). The estimated mean lifetime cost for treating bladder cancer was determined to be \$65,158 in the US and the economic burden of bladder cancer is likely to increase as survival rates increase. In addition, almost one third of the total mean costs of bladder cancer were attributed to the treatment of complications (indirect-treatment related costs) (Avritscher et al., 2006). Hence, it is important to precisely evaluate the toxic effects of treatments used for bladder cancer. The magnitude of the burdens of bladder cancer appears to be comparable in other developed countries such as Australia (Botteman et al., 2003).

Some studies have investigated the systemic and local side effects of chemotherapeutic agents on the bladder, but there is still a gap in this field which demands that more comprehensive studies be undertaken to determine the effects of these drugs on the bladder at a cellular and functional level. Hence, this study, which measured changes in urothelial and detrusor function following drug treatment, was the first to our knowledge that assessed the functional effects of the chemotherapeutic agents, doxorubicin, MMC and epirubicin on the bladder.

The Bladder

Bladder structure

The bladder is a hollow, muscular and elastic organ situated below the peritoneum. The bladder wall is composed of three distinct layers. An epithelial layer lines the internal surface of the bladder and is known as the urothelium. The next layer is known as the suburothelium and is composed of the submucosa and lamina propria which is richly supplied by blood vessels and nerves and also contains myofibroblasts. Immediately underneath this is the detrusor smooth muscle, divided into an inner (circular) and outer (longitudinal) tunica muscularis, and the outer surface is covered with adventitia. The control of normal bladder function is dependent on the functions of these layers (Figure 1.9) (Birder et al., 2010b).

The bladder serves its primary role as a short-term storage organ for urine which contains waste substances filtered from the kidneys. It is roughly spherical in shape and varies in size as it is filled with urine. Normal bladder capacity ranges between 400 and 600ml, and filling with 150-300ml triggers the first urge to urinate (GuhaSarkar and Banerjee, 2010). Maximum urine concentration in human is 1160 mOsm/kg (Verma et al., 2011).

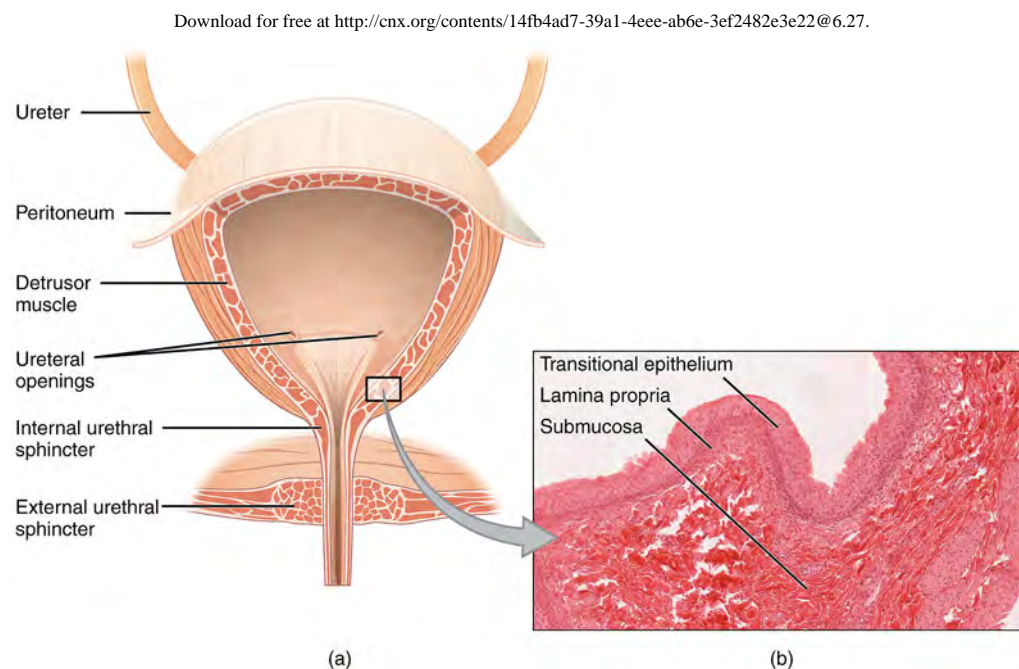


Figure 1.9: (A) Anterior cross section of the bladder. (B) The detrusor muscle of the bladder (OpenStax College, 2014).

Lower urinary system

The lower urinary system, composed of the bladder and urethra, is distinctive from other organ systems. For example, the bladder has only two modes of operation, storage and elimination of urine. Thus, many of the neural circuits that are involved in bladder control have switch-like or phasic patterns of activity, unlike tonic patterns that are characteristics of the autonomic pathways that regulate cardiovascular organs. Also, micturition (or urination) occurs voluntarily and depends on learned behaviour that develops during maturation of the nervous system, whereas many other visceral functions are regulated involuntarily. This process is controlled by the neural circuitry which is complex and highly distributed. It involves pathways at many levels of the brain, the spinal cord and the peripheral nervous system and is regulated by various neurotransmitters (Figure 1.10) (Fowler et al., 2008).

The regulation of the bladder functions such as filling, voiding and sensation of bladder fullness depends on a complex interplay between autonomic (sympathetic and parasympathetic nerves) and somatic (pudendal nerves) efferent pathways. The sympathetic innervation originates in the thoracolumbar outflow of the spinal cord, whereas the parasympathetic and somatic innervation arises in the sacral segments of the spinal cord (Figure 1.10A). In addition, afferent axons of the lower urinary tract also travel in these nerves (Fowler et al., 2008). The detrusor smooth muscles are innervated mainly by parasympathetic nerves, smooth muscles of bladder neck and urethra (internal sphincter) are innervated by sympathetic nerves and the striated skeletal muscle of external internal sphincter is innervated by somatic nerves (Chancellor and Yoshimura, 2004).

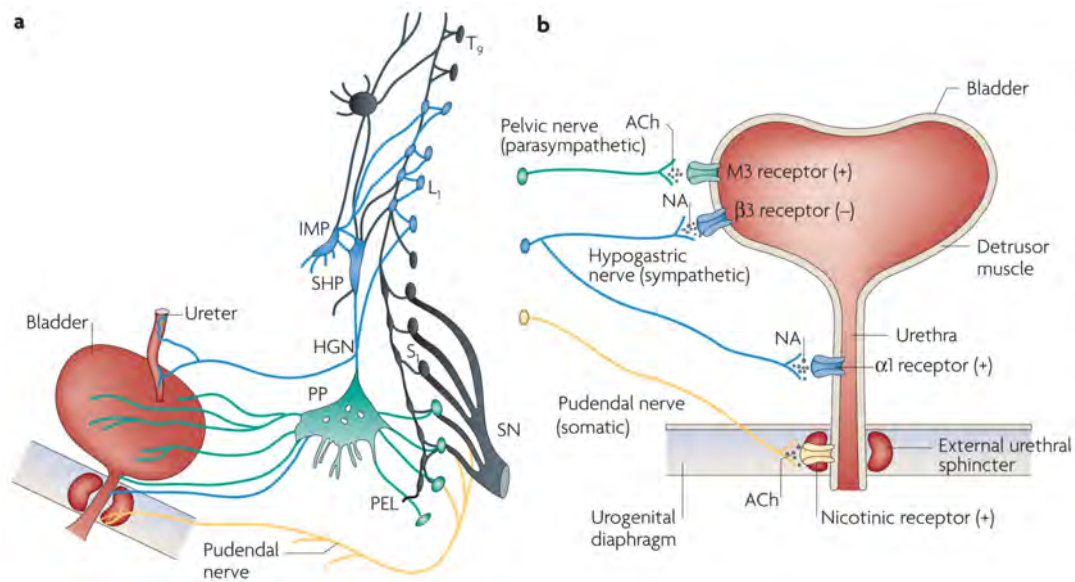


Figure 1.10: Efferent pathways of the lower urinary tract. (A) Sympathetic fibres (shown in blue) originate in the T11–L2 segments in the spinal cord and run through the inferior mesenteric ganglia (inferior mesenteric plexus, IMP) and the hypogastric nerve (HGN) or through the paravertebral chain to enter the pelvic nerves at the base of the bladder and the urethra. Parasympathetic preganglionic fibres (shown in green) arise from the S2–S4 spinal segments and travel in sacral roots and pelvic nerves (PEL) to ganglia in the pelvic plexus (PP) and in the bladder wall. This is where the postganglionic nerves that supply parasympathetic innervation to the bladder arise. Somatic motor nerves (shown in yellow) that supply the striated muscles of the external urethral sphincter arise from S2–S4 motor neurons and pass through the pudendal nerves. (B) Efferent pathways and neurotransmitter mechanisms that regulate the lower urinary tract. Parasympathetic postganglionic axons in the pelvic nerve release acetylcholine (ACh), which produces a bladder contraction by stimulating M_3 muscarinic receptors in the bladder smooth muscle. Sympathetic postganglionic neurons release noradrenaline (NA), which activates β_3 adrenergic receptors to relax bladder smooth muscle and activates α_1 adrenergic receptors to contract urethral smooth muscle. Somatic axons in the pudendal nerve also release ACh, which produces a contraction of the external sphincter striated muscle by activating nicotinic cholinergic receptors. Parasympathetic postganglionic nerves also release ATP, which excites bladder smooth muscle, and NO, which relaxes urethral smooth muscle (not shown). L1, first lumbar root; S1, first sacral root; SHP, superior hypogastric plexus; SN, sciatic nerve; T9, ninth thoracic root (Fowler et al., 2008).

Autonomic and Somatic efferent pathways

Sympathetic innervations (bladder filling)

During the filling stage, noradrenaline (NA) released from sympathetic postganglionic nerves (e.g., the hypogastric nerve) acts on α_1 -adrenoceptors (excitatory receptor) in the urethra and the bladder neck region causing contraction, which maintains closure of the bladder outlet. NA also acts on β -adrenoceptors (inhibitory receptor) on the bladder dome to cause relaxation of the detrusor muscle to prevent any increase in intravesical pressure during filling (Chess-Williams, 2002; Fowler et al., 2008). NO is co-released with NA which further enhances relaxation (Apodaca et al., 2007). During the stage of bladder filling, the parasympathetic innervation of the detrusor muscle is inhibited and the smooth and striated parts of the urethral sphincter are contracted, preventing involuntary voiding of the urine. This process is known as the 'guarding reflex' (Fowler et al., 2008) and they are triggered by afferent activity of the bladder that is conveyed through the pelvic nerves, and are organised by interneuronal circuitry in the spinal cord (de Groat, 1995; deGroat et al., 1996). Some inputs from the lateral pons, which is also known as 'pontine storage centre', appear to facilitate sphincter reflexes or play a role in involuntary sphincter control (Holstege et al., 1986). The processing of bladder sensations in human such as desire to void is thought to be mapped in insula (Craig, 2002), since brain imaging studies have shown activation of the periaqueductal gray (PAG) during bladder filling which is postulated to play a role in receiving bladder afferents and relaying them (perhaps through the thalamus) to the insula (DasGupta et al., 2007; Kavia et al., 2005). Consistent with this postulate, it was found that the insula was active in most imaging studies of urine storage (Griffiths and Tadic, 2008) and its activation was noticeably enhanced during bladder filling (Griffiths et al., 2005). The anterior cingulate cortex which also has connection with the PAG appears to determine how much attention one pays to signals coming from bladder afferents and how one reacts to them, whether by deciding to void or by recruiting mechanisms (for example, urethral sphincter contraction) that allow voiding to be postponed (Griffiths and Tadic, 2008). In addition, the frontal lobes especially the prefrontal cortex have been suggested by both clinical studies and studies from functional imaging that it might be responsible for tonic suppression of voiding that

is relaxed only when voiding is both desired and socially appropriate (Holstege, 2005; Pardo et al., 1991).

Parasympathetic innervations (bladder voiding)

It has been indicated that the circuitry, that conveys afferent nerve activity of the bladder to midbrain and pontine centres and transmits efferent signals from the pons to the sacral cord, is responsible for a switch-like function of the spinobulbospinal micturition reflex pathway that is either in complete 'off' mode during storage phase or a maximally 'on' mode during voiding phase (de Groat, 1995). During bladder filling the parasympathetic efferent pathway to the bladder, including a population of pontine micturition centre (PMC) neurons in which a spinobulbospinal pathway passes through, is turned off but at a critical level of bladder distension the afferent activity arising from tension receptors in the bladder switches the pathway to maximal activity (de Groat et al., 1998; De Groat et al., 1982; Sugaya et al., 2005). During emptying, a reduction in sympathetic and an increase in parasympathetic activity occurs to reverse the functions of the bladder dome and neck. Parasympathetic postganglionic nerves release the cholinergic transmitter, acetylcholine (ACh) which is the major excitatory mechanism in the human bladder (Figure 1.10B) act on muscarinic receptors (G-protein coupled), causing detrusor contraction and consequent urinary flow (Andersson and Arner, 2004). It is mediated principally by the M_3 muscarinic receptor, although bladder smooth muscle also expresses M_2 muscarinic receptors (Matsui et al., 2002). Activation of muscarinic receptors on parasympathetic nerve terminals at the neuromuscular junction and in the parasympathetic ganglia can enhance transmitter release through muscarinic M_1 receptors or suppress transmitter release through muscarinic M_4 receptors, depending on the intensity of the neural firing (Somogyi et al., 1998). ACh also acts on nicotinic receptors (ionotropic) which mediate fast synaptic transmission in autonomic ganglia, and activation of nicotinic receptors in parasympathetic bladder neurons causes detrusor muscle contraction. Autonomic ganglia contain transcripts for α_3 , α_4 , α_5 , α_7 , β_2 , and β_4 nicotinic subunits, which can assemble to form multiple receptor subtypes. It was reported that α_3 and β_4 subunits have the higher expression levels. However, the exact nicotinic receptor subunit composition in bladder ganglia is unknown (De Biasi et al., 2000). Adenosine triphosphate (ATP) which is a non-cholinergic excitatory transmitter is co-released with ACh following muscarinic nerve

stimulation. ATP acts on P2X purinergic receptors in the detrusor muscle, initiating micturition while ACh maintains bladder voiding (Burnstock, 2014). Muscarinic receptor stimulation induces NO release from the parasympathetic nerves to cause relaxation of the urethral smooth muscle, allowing bladder voiding to occur with little increase in intravesical pressure (Andersson and Arner, 2004; Chess-Williams, 2002). Prostanoids such as PGE₂ are also released from the urothelium and are thought to play a role in stimulating the micturition reflex (Aoki et al., 2009).

Somatic innervations

The striated skeletal muscles of the external urinary sphincter and pelvic floor are innervated by somatic nerves. The efferent motor nerves arise in the lateral border of the ventral horn in sacral cord segments S₂ to S₄, located in Onuf's nucleus (Fowler et al., 2008; Thor et al., 1989; Yoshimura and de Groat, 1997), and reach the periphery through the pudendal nerve to release ACh. The ACh acts on nicotinic receptors (the exact subunit composition unknown) in the striated muscle to cause muscle contraction, maintaining closure of the external urinary sphincter (Blaivas, 1982; Thor et al., 1989; Yoshimura and de Groat, 1997).

Neurotransmitters

It can be seen from the above that neurotransmitters play an active role in regulating functions of the lower urinary tract. Other neurotransmitters including glutamic acid, tachykinins and pituitary-adenylate-cyclase-activating polypeptide appears to have an excitatory effect on contraction (Matsuura et al., 2002; Sugaya et al., 2005), and GABA (γ -aminobutyric acid), glycine and opioid peptides (enkephalins) which are inhibitory amino acids exert a tonic inhibitory control in the PMC and regulate bladder capacity (Mallory et al., 1991). Some transmitters have either inhibitory or excitatory effects depending on the type of receptor that is activated and these include dopamine, serotonin (5-HT) and non-opioid peptides (Andersson and Pehrson, 2003).

Bladder sensations (afferent pathway)

Information about the degree of bladder distension is carried to the spinal cord by the pelvic and hypogastric nerves, whereas sensory input from the bladder neck and urethra is conveyed in the pudendal and hypogastric nerves. Myelinated (A δ) and unmyelinated (C) axons form the afferent components of these nerves and the cell bodies of these fibres are located in the dorsal root ganglia (DRG) at the level of S2-S4 and T11-L2 spinal segments (Fowler et al., 2008). Information about bladder filling is conveyed by the A δ -fibres which respond to passive distension and active contraction (Janig and Morrison, 1986). The C-fibres which is also known as ‘silent’ C-fibres are insensitive to bladder filling under physiological conditions and respond predominantly to noxious stimuli such as chemical irritation or cooling (Fall et al., 1990; Habler et al., 1990).

Furthermore, studies have shown that non-neuronal cells such as urothelial cells and suburothelial myofibroblasts also play a part in regulating bladder functions and these will be discussed further in following sections.

Detrusor muscle

The detrusor muscle, which is also known as muscularis propria, is a layer which consists of smooth muscle fibres arranged in spiral, longitudinal and circular bundles that form the wall of the bladder (Marieb and Hoehn, 2010).

Human detrusor muscle is enriched with muscarinic receptors; the majority of which are the M₂ subtype whereas the remaining minority are the M₃ subtype (Hegde et al., 1997). The ratio of M₂ to M₃ muscarinic receptors varies among the species but human is known to have a ratio of 3:1 (Wang et al., 1995). *In vitro* functional responses to muscarinic receptor stimulation which leads to bladder contraction is mediated via the minor population of M₃ muscarinic receptor in pig (Sellers et al., 2000) and human bladder (Chess-Williams et al., 2001). The role of the larger population of M₂ muscarinic receptors is not fully understood, however they are assumed to be responsible for inhibiting the cAMP-induced relaxations of the detrusor muscle to allow the contraction to occur (Yamanishi et al., 2002a; Yamanishi et al., 2002b). The roles of other muscarinic receptors M₁, M₄ and M₅ in bladder function are as yet not well understood.

There is increasing evidence suggesting that β -adrenoceptors (β -ARs) are abundant in the detrusor muscle of the bladder of various species (Limberg et al., 2010). The β -ARs can be subclassified into β_1 -, β_2 -, and β_3 and the β -AR subtype mediating relaxation of detrusor muscle appears to be species dependent (Limberg et al., 2010; Masunaga et al., 2010). In most species, including rabbit and rat, detrusor relaxation is mediated predominantly through the action of the β_2 -ARs. However, in humans, the β_3 -AR subtype predominates and mediates detrusor relaxation (Limberg et al., 2010; Masunaga et al., 2010; Tyagi et al., 2009).

Previously, bladder function was believed to be largely dependent on the innervations of the autonomic system through muscarinic and adrenergic receptors on detrusor muscle. However, there is increasing evidence suggesting that the urothelium and suburothelium also play an important role in modulating normal function of the bladder.

Lamina propria (suburothelium)

Immediately beneath the urothelium is a layer known as the lamina propria which contains a number of cell types including afferent and efferent nerve terminals (Fry et al. 2007). The different types of afferent fibres have been shown to be present in the bladder including unmyelinated C fibres and myelinated A δ fibres (Birder et al., 2010a; Gillespie et al., 2006). Conceptually, A δ fibres are known to carry information regarding physiological stretch or contraction of the bladder wall, whereas C fibres are associated with volume or higher threshold sensations (pain) (Morrison, 1999).

In both humans and animals, the cells with similar morphological characteristics to those of myofibroblasts or interstitial cells (ICs) have been recently reported within the lamina propria layer (Brading and McCloskey, 2005; Fry et al., 2007; Ost et al., 2002; Sui et al., 2004). Myofibroblasts in lamina propria consist of long spindle-shaped cells and are connected to each other through gap junctions (Fry et al., 2007). These cells also have close contacts with nerves, display numerous receptors and ion channels (i.e. epithelium sodium channel (ENaC), purinergic, cholinergic and adrenergic) and can respond to neurotransmitters such as ATP released from nerves or the urothelium (Birder et al., 2010b; Fry et al., 2007). These suggest that the

function of myofibroblasts in lamina propria is to act as intermediaries in urothelial-nerve interactions potentially via their ability to propagate signals over many cell lengths (Fry et al., 2007). However, their role within the bladder has yet to be elucidated.

Urothelium

The urothelium is composed of basal cells connected to the basement membrane, intermediate cells and the most superficial layer, ‘umbrella cells’ (Figure 1.11B) (Khandelwal et al., 2009).

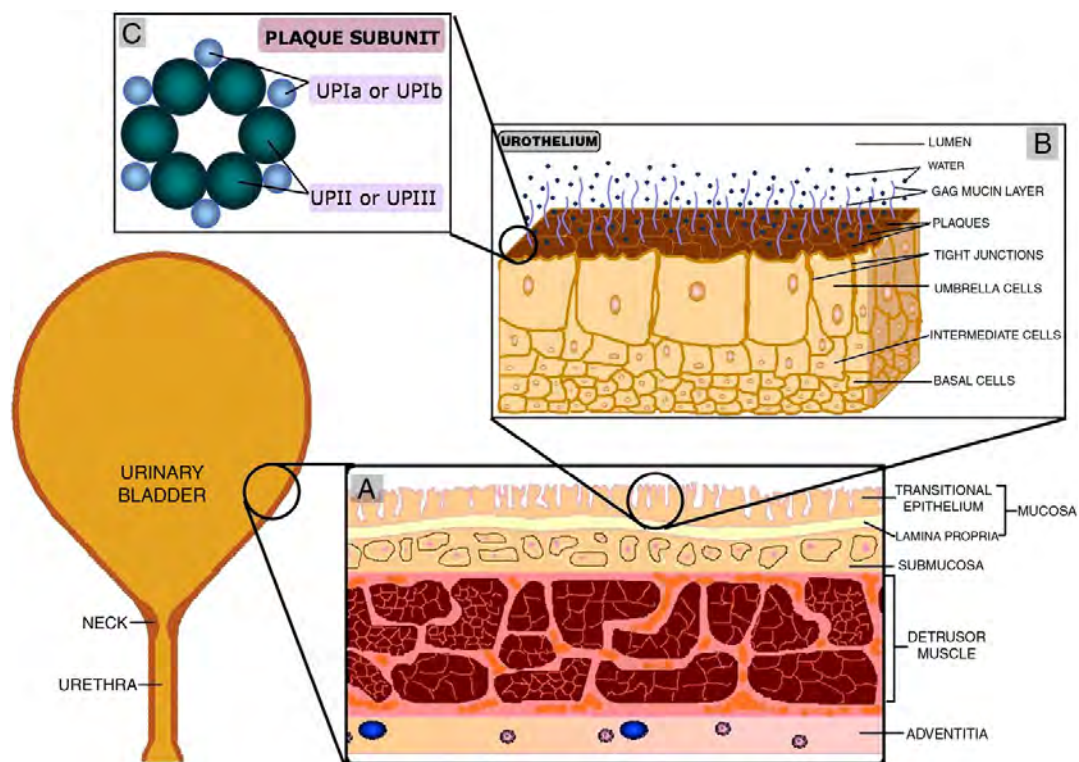


Figure 1.11: Structure of the urinary bladder wall and urothelium. (A) Detailed view of the layers of the bladder wall showing the epithelial, mucosal and muscle layers. (B) Layers of cells in the transitional epithelium, or urothelium, of the bladder wall, showing the characteristic umbrella cells joined by tight junctions and covered by plaques and a mucin layer. Each plaque has 1000 subunits. (C) Uroplakins (UPIa, UPIb, UPII and UPIII) arranged to form a single plaque subunit (GuhaSarkar and Banerjee, 2010).

Since, the urine contains waste substances; the urothelium forms a functional distensible barrier that prevents unregulated exchange of substances between the urine and the systemic circulation (GuhaSarkar and Banerjee, 2010). This task is achieved by the umbrella cells joined by high-resistance tight junction and with an

apical surface covered by densely packed plaques (Figure 1.11B). During bladder filling, the umbrella cells become flat and squamous by vesicular traffic (i.e. exocytosis/endocytosis) and this shape change allows the bladder to fill with urine without compromising barrier function (Birder et al., 2010b). The barrier property of urothelium is further enhanced by the presence of sulphated polysaccharide glycosaminoglycans (GAGs) adherent to the luminal side. GAGs effectively block solutions and solutes from penetrating and reaching underlying layers by preventing adhesion of foreign particles (act as a defensive mechanism against infection) (Birder et al., 2010b; Birder et al., 2010g; GuhaSarkar and Banerjee, 2010; Khandelwal et al., 2009).

This barrier of umbrella cells with their tight junctions, densely packed plaques and GAGs also prevent effective diffusion of drugs used for intravesical therapy to treat bladder cancer. Hence, the permeability of drug solutions into the urothelium depends on the degree of structural or chemical disruption occurring on GAGs and umbrella cells (GuhaSarkar and Banerjee, 2010).

Increased permeability to intravesical drugs may occur in neoplastic urothelial cells due to dedifferentiation and impaired production of membrane plaques, GAGs and tight junctions. Drug permeability may also be facilitated by cystitis and transurethral resection (Badalament and Farah, 1997). Since the urothelium serves as an effective barrier only a small percentage of drug is absorbed into the systemic circulation. This is evident in an *in vitro* study conducted by Wientjes, Badalament, Wang, Hassan and Au (1993) which used human bladders from bladder cancer patients who received instillation of mitomycin C (20mg/40mL) at the time of radical cystectomy. This study has shown that mitomycin C concentration dropped by 35-fold across the urothelium. The mean plasma concentrations were 0.003, 0.1, and 0.4% of the mean concentration in urine, urothelium and the averaged bladder tissue concentrations, respectively. Further an *in vitro* study (using human bladder from bladder cancer patients who received instillation of 40mg/20mL of doxorubicin prior to radical cystectomy) conducted by Wientjes et al. (1996) has shown that the doxorubicin concentration dropped by 32-fold across the urothelium, and declined semi-logarithmically with respect to depth in the capillary-perfused tissues beneath the urothelium with a 50% decrease over about 500µm. The plasma concentrations were

0.02, 0.03, 0.05, 0.27 and 0.67% of concentrations in the tumours, urothelium, lamina propria, superficial and deep muscle layers, respectively.

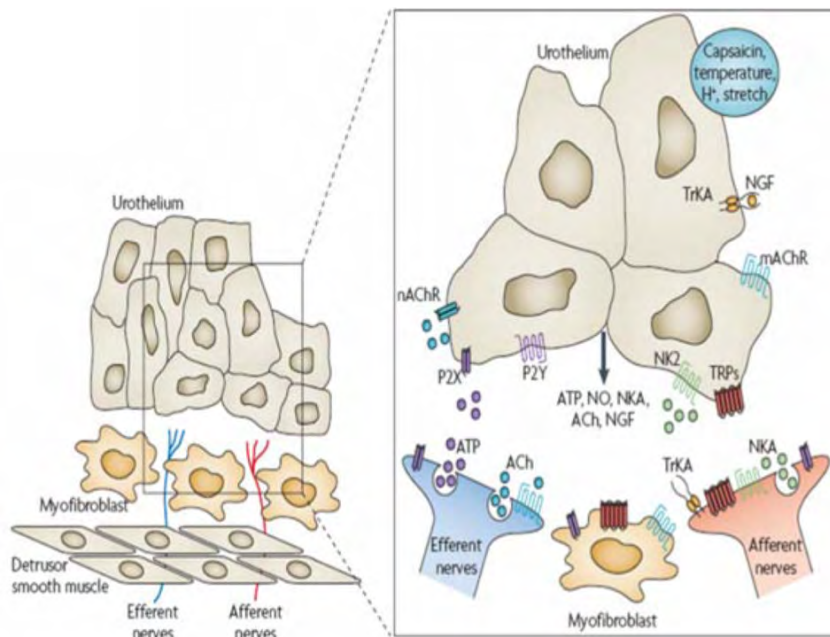


Figure 1.12: Illustration of the possible interactions between the urothelium, bladder afferent and efferent nerves, myofibroblasts and detrusor muscle (Birder, 2010).

While it was originally believed that the urothelium served solely as an effective barrier to limit the absorption of molecules and ions into the systemic circulation, it is now recognised as a more active tissue with specialised sensory properties that enable the urothelium to act as a primary transducer of some physical and chemical stimuli, and able to communicate with underlying cells including nerves, connective and muscular tissues, and inflammatory cells (Birder, 2013; Fowler et al., 2008). The urothelium is able to respond to a wide range of physical stimuli including changes in bladder pressure, tension in the urothelium or bladder wall, torsion, geometrical tension and ranges of urine composition as well as tonicity during bladder filling and voiding. Additional lines of studies in human and animals suggest that urothelial cells are able to respond to not only physical but also to chemical stimuli (Birder, 2013; Birder and Wyndaele, 2013). There is evidence that both afferent and autonomic afferent nerves are located in close proximity, with some penetrating the urothelium. These nerves can be activated by a range of transmitters and mediators released in part by the urothelium (Birder and Wyndaele, 2013). Various stimuli can lead to a number of outputs from the urothelium including the secretion of numerous transmitters and mediators, such as neurotrophins, peptides, ATP, acetylcholine, noradrenaline, prostaglandins, prostacyclin, NO, cytokines and an unidentified factor

that inhibit detrusor contraction (Figure 1.12) (Apodaca et al., 2007; Birder, 2013; Fowler et al., 2008; Hawthorn et al., 2000). The urothelium also expresses various receptors and ion channels including nicotinic, muscarinic, tachykinin, adrenergic, cholinergic, bradykinin, epithelium sodium channel (ENaC) and transient-receptor-potential vanilloid receptors (such as TRPV1) (Birder, 2013; Fowler et al., 2008). More detailed information on release of urothelial factors will be discussed in the following sections.

Urothelial receptors and mediators

Acetylcholine (ACh)

The discovery of muscarinic and nicotinic receptors on the urothelium has attracted further interest in the role of ACh in bladder function as a chemical mediator of neural-urothelial interactions (Birder, 2010). Muscarinic receptors are primarily known for their role in voiding but there is evidence showing that the urothelium expresses the full complement of muscarinic receptors (M_1 - M_5) as well as the enzymes and transporters necessary for the synthesis, release and metabolism of ACh.

A study by Lips et al. (2007) reported that the enzyme involved in urothelial ACh synthesis was not choline acetyltransferase (ChAT) but carnitine acetyltransferase (CarAT). The exact mechanism of urothelial-derived ACh release is as yet unknown but it appears not to be vesicular acetylcholine transporter (VACHT) which is responsible for transporting neuronal ACh, since it was absent from the urothelium. Also, the organic cation transporters (OCTs) 1 and 3, which can transport ACh in either direction across cell membranes, even though they are present in mouse and human urothelium, were demonstrated to be not involved in ACh release (Lips et al., 2007). A recent study by McLatchie et al. (2014) has suggested potential involvement of cystic fibrosis transmembrane conductance regulator (CFTR) in ACh release. Studies have shown that ACh is released from the urothelium in response to stretch (mechanical stimulation) as well as chemical stimulation (Birder, 2010). Once released, urothelial-derived ACh is likely to exert effects on a number of sites. ACh may act in a paracrine manner to stimulate nicotinic and/or muscarinic receptors on detrusor muscle, myofibroblasts and afferent nerves, or in an autocrine manner to stimulate urothelium associated muscarinic receptors (Birder, 2010; Hanna-Mitchell

et al., 2007). The role of cholinergic signalling in the urothelium is still being identified. However, a study by Kanai et al. (2007) which applied muscarinic receptor agonist to strips of rat bladder tissue induced membrane-potential transients and Ca^{2+} transients that begin near the urothelial-suburothelial interface and then spread to the detrusor smooth muscle, raising the possibility that the urothelium could regulate the generation of spontaneous, non-voiding contraction in the bladder.

Various cell studies have suggested that both M_1 -like (M_1 , M_3 and M_5) and M_2 -like muscarinic receptors (M_2 and M_4) can be involved in tissue remodelling and promote cell growth and proliferation (Belo et al., 2011; Gosens et al., 2004; Gosens et al., 2006; Matthiesen et al., 2007; Tong et al., 2009) and those of the M_1 -family can even act as conditional oncogenes (Gutkind et al., 1991). Such growth-promoting and pro-survival effects of muscarinic receptors may involve the extracellular signal-regulated kinases (ERK) and phosphoinositide (PI)-3 kinase/Akt pathway (Wu and Wong, 2006). Also, a study conducted by Arrighi et al. (2011) demonstrated that cell proliferation of human urothelium (UROtsa cell line) was stimulated by the cholinergic receptor agonist, carbachol via pathway involving muscarinic M_3 receptors, ERK and PI-3 kinase supporting the cell growth and proliferation promoting effects of muscarinic receptors in the urothelium. Hence, bladder remodelling via cholinergic system might be responsible for urothelial hypertrophy which can develop in pathological settings such as bladder outlet obstruction.

It is well established that patients with overactive bladder (OAB) can be effectively treated with muscarinic receptor antagonists. This agent enhances the storage phase of micturition by targeting post-junctional excitatory muscarinic receptors (M_2 and M_3) in the detrusor smooth muscle to silence parasympathetic innervations (Hegde, 2006; Pathak and Aboseif, 2005; Staskin and MacDiarmid, 2006). Since, the urothelium also expresses the full complement of muscarinic receptors, it has attracted interest in the role of urothelial derived ACh in the OAB (Yoshida et al., 2006). Increased cholinergic afferent effects, specifically sensitisation of acetylcholine-evoked signalling in the urothelium have been reported in patients with OAB (Yoshida et al., 2010). It is postulated that upregulation of the cholinergic targets (possibly muscarinic M_2 receptors on afferent nerve terminals) is responsible for this rather than just increased acetylcholine production itself (Matsumoto et al., 2010). In contrast, decreased expression of muscarinic receptors in urothelial and

suburothelial cells in OAB patients has been reported (Mansfield et al., 2007; Mukerji et al., 2006). In addition, upregulation of the urothelial muscarinic M₁ and M₅ receptors in cystitis has been shown, suggesting that alterations in the urothelial expression of muscarinic receptors may take part in pathophysiology of cystitis (Giglio et al., 2008; Giglio et al., 2005). Furthermore, activation of the mucosal muscarinic receptors has been reported to be coupled with NO release, and this pathway seems to be altered in the inflamed urinary bladder (Andersson et al., 2012; Andersson et al., 2008).

In addition, there is growing evidence showing that the urothelium expresses multiple nicotinic receptors (Beckel and Birder, 2012; Beckel et al., 2006; Bschleipfer et al., 2007; Zarghooni et al., 2007). A study using human bladder mucosal biopsies has demonstrated the expression of nicotinic α -subunits 7, 9 and 10 (rank order of $\alpha 7 > \alpha 10 > \alpha 9$) in the human urothelium (Bschleipfer et al., 2007). Furthermore, study by Beckel & Birder (2012) which used rat urothelial cells, when stimulated with $\alpha 7$ nicotinic receptor agonist, increased intracellular Ca²⁺ through internal stores and decreased basal ATP release suggesting inhibitory effect of an $\alpha 7$ receptor activation on bladder reflexes. Stimulation with an $\alpha 3$ nicotinic receptor agonist also increased intracellular calcium through extracellular influx as well as basal ATP release, indicating possible excitatory effect. When the cells were pretreated with an $\alpha 7$ agonist, the cellular effects of $\alpha 3$ stimulation previously observed was blocked, suggesting interactions between nicotinic subtypes whereby activation of $\alpha 7$ receptors inhibits the response to subsequent activation of $\alpha 3$ receptors, preventing the increase in calcium concentration. These observations suggest that urothelial nicotinic acetylcholine receptors may modulate ATP release, indicating a role in nociceptive signalling in the lower urinary tract.

Adenosine Triphosphate (ATP)

ATP was the first neurotransmitter shown to be released directly from the urothelium. Since the first discovery of ATP release from non-neuronal sources in the rat bladder by Ferguson et al. (1997), there is now abundant evidence supporting an important role of ATP release from the urothelium in sensory and motor functions of the bladder.

ATP is released from both the apical and basolateral epithelial surfaces in response to bladder stretch during filling (Wang et al., 2005). Various studies have shown stretch-induced ATP release from the urothelium in both tissues (Kumar et al., 2004; Kumar et al., 2007; Munoz et al., 2011; Sadananda et al., 2009) and cells (Kang et al., 2013; Mansfield and Hughes, 2014a, e). The ATP released from the urothelium can act in both an autocrine and a paracrine manner. The main source of ATP release in the bladder is the urothelium and not the detrusor muscle (Kumar et al., 2004; Sadananda et al., 2009). The mechanism of urothelial-derived ATP release is not fully understood yet. Studies have shown that involvement of extracellular calcium is contradictory, since removing extracellular calcium had no effect in one study (Sui et al., 2014) but increased (Ferguson et al., 1997; Young et al., 2012) or decreased (Birder et al., 2003; Cheng et al., 2011; Olsen et al., 2011) ATP release in other studies. One of the potential mechanisms appears to be vesicular release (Birder et al., 2003; McLatchie and Fry, 2014; Sui et al., 2014; Wang et al., 2005) and another likely route includes connexin or pannexin hemichannels (McLatchie and Fry, 2014; Timoteo et al., 2014; Wang et al., 2005), transient receptor potential channels (TRPV) 1 or 4 (Mochizuki et al., 2009; Wang et al., 2005), ATP-binding cassette (ABC) transporters (Wang et al., 2005) or mechanosensitive channels (Birder et al., 2003; Dunning-Davies et al., 2013). The urothelial-derived ATP can enhance its own release from urothelial cells by binding to urothelium-associated purinoceptors, P2X₂ and P2X₃. The binding of ATP to purinergic receptors can also alter epithelial functions via exocytosis which can adjust the apical surface area during bladder filling (Birder, 2010; Khandelwal et al., 2009; Wang et al., 2005).

The ATP released from the urothelium can directly depolarize and initiate firing in sensory nerves by activating ionotropic P2X channels or metabotropic P2Y receptors on afferent nerves as well as the urothelium to stimulate intracellular second messenger pathways that in turn modulate other ion channels (Khandelwal et al., 2009; Tominaga et al., 2001).

As the epithelium stretches during bladder filling, ATP is released and may stimulate the P2X receptors present on urothelial and suburothelial sensory A δ and small diameter C fibres, stimulating the afferent arm of the micturition reflex and giving rise to the perception of pain respectively; therefore serving as a mediator for the degree of distension of bladder (Cockayne et al., 2000; Kumar et al., 2010; Kumar et

al., 2007; Souslova et al., 2000). The role of ATP release from the urothelium has attracted attention because intravesical administration of ATP induced detrusor overactivity by stimulating the purinergic receptor P2X₃ or P2X_{2/3} on afferent nerves (Rong et al., 2002). In addition, reduced bladder activity and inefficient voiding was observed in mice that lack P2X₃ receptors suggesting that activation of P2X₃ receptors on bladder afferent nerves by ATP released from the urothelium is essential for normal bladder function (Cockayne et al., 2000). Hence, the purinergic P2X transmission has a role in the initiation of detrusor contraction, thereby initiating bladder voiding, whereas cholinergic transmission maintains bladder contraction and flow (Kumar et al., 2004; Stevens et al., 2007).

To date, eight P2Y receptors (P2Y₁, P2Y₂, P2Y₄, P2Y₆, P2Y₁₁₋₁₄) have been identified with P2Y₁, P2Y₂, P2Y₄, and P2Y₆ receptors detected in peripheral sensory neurons located in dorsal root and nodose ganglia (Gerevich and Illes, 2004; Ruan and Burnstock, 2003). It has been reported that P2Y₁, P2Y₂, P2Y₄ and P2Y₁₁ are also expressed on urothelial cells (Birder et al., 2004; Chopra et al., 2008; Shabir et al., 2013). The P2Y receptors respond to endogenous purine and pyrimidine nucleotides (ATP, ADP, UTP, UDP) released into the extracellular environment from various tissues (Lazarowski and Boucher, 2001; Lazarowski and Harden, 1999; von Kugelgen, 2006). While function of these receptors in the bladder has not been elucidated yet, there is some evidence supporting involvement of P2Y receptors in mechanosensation and or/ nociception under normal and pathological conditions. A study by Fry et al. (2012) reported increased spontaneous activity in rat bladder sheets after exogenous application of P2Y receptor agonist (ADP and UTP). Another study on mouse bladder sensory neurons has demonstrated increased excitability of bladder neurons by UTP (a P2Y₂ and P2Y₄ agonist) via depolarizing resting membrane potential, increasing action potential firing, and facilitating purinergic (P2X) currents (Chen et al., 2010). In addition, intravesical instillation of a P2Y₆ selective agonist in a rat cystometry model induced bladder overactivity characterised by increased urinary frequency, and also increased ATP release in the voided fluid (Timoteo et al., 2014). Furthermore, decreased expression of P2Y₂ receptor was reported in a feline model of interstitial cystitis (IC) (Birder et al., 2004). Thus, P2Y receptors might be also contributing to hyperexcitability and increased firing of bladder afferents that are associated with urinary urgency, frequency, and

pain, all of which are features of OAB and IC (also known as painful bladder syndrome (PBS)) (Nazif et al., 2007).

Although, purinergic control is less important than that of cholinergic in normal conditions, it has increasingly been accepted as having more significant role in sensory abnormalities observed in a number of bladder disorders such as IC and OAB. Various *in vitro* studies using animal models of OAB and IC have suggested that increased urothelial ATP release from damaged or sensitised cells in response to injury or inflammation might be the contributing factor for triggering increased bladder activity and painful sensations via excitation of purinergic (P2X) receptors on sensory fibers (Khera et al., 2004; Munoz et al., 2011; Smith et al., 2005; Sun et al., 2001). Not only the alterations in the urothelial ATP release but also changes such as inhibition of ecto-ATPase activity (Nishiguchi et al., 2005) as well as increased density of P2X receptors (Moore et al., 1992) were observed in OAB, which renders detrusor muscle to be more responsive to ATP (Santoso et al., 2010).

Prostaglandin E₂

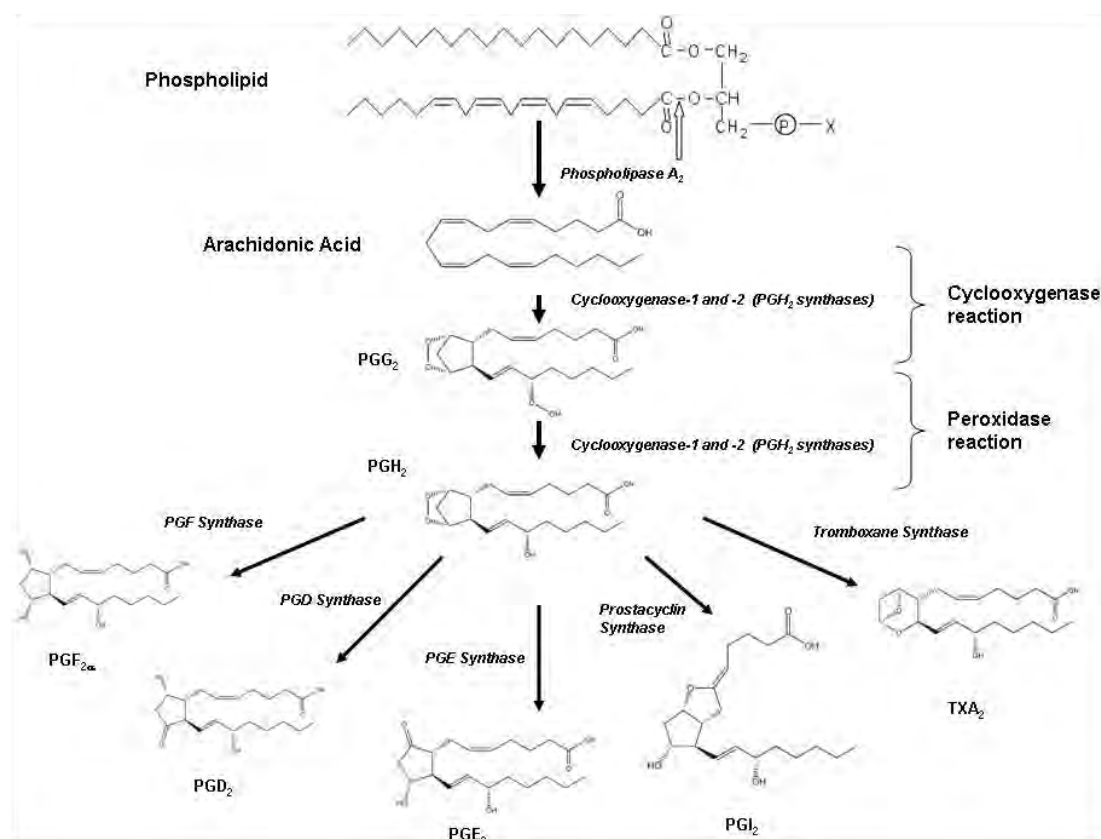


Figure 1.13: Synthesis of prostanooids from arachidonic acid (Sorokin, 2011).

Prostaglandins, along with thromboxanes and prostacyclins are the products of the cyclooxygenase (COX) pathway, collectively known as the prostanoids. There are two isoforms of cyclooxygenase enzymes, COX-1 and COX-2. Both cyclooxygenase isoforms metabolize the fatty acid arachidonic acid into first PGG_2 by cyclooxygenase activity of COX and PGG_2 is further metabolised into PGH_2 by peroxidase activity of COX. Then, PGH_2 is converted into various prostanoids by respective synthases (Figure 1.13) (Rahnama'i et al., 2012).

There are four primary prostaglandins synthesised and released in the bladders of various species during detrusor contraction (Bultitude et al., 1976; Khalaf et al., 1980; Klarskov, 1987) and under basal physiological conditions (Brown et al., 1980; Poggesi et al., 1980). These includes prostaglandin E_2 (PGE_2), prostacyclin I_2 (also known as prostaglandin I_2 ; PGI_2), prostaglandin D_2 (PGD_2) and prostaglandin $\text{F}_{2\alpha}$ ($\text{PGF}_{2\alpha}$). They are ubiquitously produced and act as an autocrine and paracrine lipid mediators to maintain local homeostasis in the body. However, prostaglandin production increases dramatically during an inflammatory response (Ricciotti and FitzGerald, 2011). In the human bladder, the major prostaglandin is PGI_2 , followed by PGE_2 and $\text{PGF}_{2\alpha}$ (Jeremy et al., 1984). Both COX-1 and COX-2 are expressed in the bladder. The expression of COX-1 by the basal layer of the urothelium (and, to a lesser extent, the cells of the intermediate urothelial layer) indicates that prostaglandins are synthesised and released from these sites (de Jongh et al., 2009; Rahnama'i et al., 2010). The detrusor smooth muscle might also synthesize prostaglandins as they also express both COX-1 and COX-2 (Klausner et al., 2011). PGE_2 synthesis can be initiated by various factors such as stretch, nerve stimulation, injury, exposure to ATP and other inflammatory mediators (Dveksler et al., 1989; Khan et al., 1998).

The exact role of urothelial-derived PGE_2 is not elucidated yet but various studies have suggested excitatory effects on bladder contractility via modulation of nerve and detrusor functions (Apodaca et al., 2007; Birder, 2005). Also, PGE_2 has been discussed extensively as one of the contributing factors that may cause urinary bladder overactivity. A study using mice has demonstrated that overexpression of PGE_2 in the bladder stimulates the micturition reflex through activation of C-fibres (Aoki et al., 2009; Maggi et al., 1988a). Inhibition of COX-2 resulted in improved storage function in rats with detrusor overactivity, indicating potential stimulatory

effects of PGE₂ on bladder micturition reflex (Yokoyama, 2010b). Also, administration of indomethacin (COX inhibitor) caused a reduction in tone and loss of spontaneous contractile activity of the isolated bladder (Bultitude et al., 1976). In addition, when PGE₂ was instilled intravesically in the conscious, catheterised rats, micturition was facilitated and an increased basal intravesical pressure was generated (Ishizuka et al., 1995). On detrusor smooth muscle strips of mouse, PGE₂ also increased the tone and caused phasic contractions of detrusor muscle (Kobayter et al., 2012). In humans, intravesical administration of PGE₂ caused detrusor overactivity, urgency and decreased bladder capacity (Schussler, 1990). Furthermore, local PGE₂ production was reported to be elevated in the bladders of animals with overactive detrusor muscles (Hu et al., 2003; Park et al., 1999) as well as patients with OAB (Kim et al., 2005; Kim et al., 2006a).

There are four prostaglandin EP receptors (EP1-EP4) (Alexander et al., 2008) that PGE₂ can act on and it mediates different effects depending on the receptors (Negishi et al., 1995). In general, stimulation of EP1 and EP3 appears to be responsible for causing contraction of the detrusor smooth muscle, whereas stimulation of EP2 and EP4 are thought to induce muscle relaxation (Coleman et al., 1994; Ikeda et al., 2006). Increased bladder capacity and reduced voiding efficiency were observed in rats by EP1 receptor antagonist (Maggi et al., 1988f). Also, EP1 knockout (KO) mice have shown that PGE₂ is not essential for normal micturition but does play a role for EP1 in detrusor overactivity (Schroder et al., 2004). The dual EP1 and EP2 receptor antagonist decreased the amplitude of detrusor contraction in isolated human bladder (Palea et al., 1998), indicating possible inhibitory role of EP2 receptor. In rats, reduced functional bladder capacity was observed with EP3 receptor agonist; whereas inhibition of this receptor resulted in opposite effect (Jugus et al., 2009). Also, diminished PGE₂-mediated bladder hyperactivity was observed in EP3 receptor knockout mice and enhanced bladder capacity was also observed under basal conditions (McCafferty et al., 2008). Study of rats with bladder outlet obstruction by Beppu et al. (2011) has shown that activation of EP4 receptor using agonist suppressed detrusor contraction and afferent nerve activity, suggesting possible inhibitory role of EP4 receptor on bladder contractility. However, in contrast, upregulation via increased expression of EP4 receptors exacerbated cyclophosphamide-induced OAB in rats (Chuang et al., 2010).

Urothelial-derived inhibitory factor (UDIF)

An unidentified substance is released from the healthy urothelium which diminishes contractility of detrusor smooth muscle. As a result of these unknown substances, bladder overactivity and consequent involuntary urine loss are prevented (Chaiyaprasithi et al., 2003; Hawthorn et al., 2000; Templeman et al., 2002). Research has shown that removal of the urothelium results in a significant increase in the detrusor muscle contractile response and further studies provided strong evidence of urothelium releasing a soluble factor that exerts an inhibitory effect on the contractility of the bladder detrusor muscle (Fovaeus et al., 1998, 1999; Hawthorn et al., 2000).

The urothelial-derived inhibitory factor or UDIF has not been identified at the present time. NO, its metabolite nitrite or P1 purinoceptor agonist like adenosine are unlikely to be the unknown factor or involved in the release of the factor from the urothelium (Guan et al., 2014; Hawthorn et al., 2000). However, other purines such as ATP and related nucleotides, due to their significant release from the urothelium (Munoz et al., 2010; Sui et al., 2014), still remain as potential candidates for UDIF. A study by McMurray et al. (1998) first reported the possibility of relaxant effect of ATP in which both contractile and relaxant effects of ATP in urothelium denuded detrusor strips were observed. Expression of purinergic P2X and P2Y receptors on detrusor smooth muscle are suggested to participate in, respectively, contractions and relaxations (Chopra et al., 2008; Elneil et al., 2001; Fry et al., 2010). Also, a recent study by Santoso et al. (2010) supported the potential inhibitory role of urothelial ATP in mediating detrusor smooth muscle contractility, which may be impaired in diseased bladders such as OAB syndrome. In addition, cyclooxygenase (COX) products such as prostaglandins and prostacyclin, which are released from the urothelium, are not involved in the inhibition of detrusor muscle contraction (Guan et al., 2014; Hawthorn et al., 2000). Furthermore, UDIF is unlikely to be catecholamine, gamma aminobutyric acid (GABA), or an apamin-sensitive response to EDHF (Hawthorn et al., 2000).

Effect of chemotherapy on urothelial factors and detrusor function

The effects of intravesical chemotherapeutic drugs on normal urothelial and detrusor function is not understood. Given the immediate contact between high concentration of these agents and the urothelium it is likely that release of urothelial factors (Ach, ATP, NO, PGE₂ and UDIF) is affected following treatment. This leads to one of the major aims of this study.

The only study to date that has assessed how intravesical chemotherapeutic agents affect release of urothelial mediators was conducted for my honours degree (2012). My study demonstrated that a common intravesical chemotherapeutic agent, doxorubicin altered the release of urothelial mediators including Ach, ATP and PGE₂ from human urothelial cell line (RT4). Immediately following treatment, stimulated ATP release was inhibited at doxorubicin concentrations $\geq 1\mu\text{g/ml}$ and showed partial recovery at 24 hour. Immediately following doxorubicin treatment, basal Ach release was increased by doxorubicin at its clinical concentration (1mg/ml), while a concentration-dependent decrease in stimulated Ach release was observed. Twenty four hours after treatment, basal Ach release was increased in culture treated with 0.01mg/ml doxorubicin while stimulated Ach release remained depressed. A significant increase in PGE₂ release was observed in cells immediately and 24 hours after treatment with doxorubicin (Kang et al., 2013). In addition, the detrusor muscle was not directly affected by doxorubicin treatment (exposure to 1mg/ml for 1 hour), however the efferent nerves were sensitised or muscarinic M₂ receptors were inhibited by doxorubicin, resulting in enhanced contractility of doxorubicin treated porcine detrusor muscle in response to electrical field stimulation (Kang et al., 2015).

The findings of this study raised the possibility of other intravesical chemotherapeutic agents such as mitomycin C and epirubicin could also exert alterations in urothelial mediators and detrusor function. Differences in incidence of reported side effects between drugs may also be explained by extent of changes in urothelial function observed.

Aims and Hypothesis

While there is an extensive research effort focused on enhancing the toxicity of intravesical cytotoxic agents, there have been few studies that have assessed their effects on normal bladder function. Some studies have investigated the systemic and local side effects of chemotherapeutic agents on the bladder, but there is still a gap in this field which demands that more comprehensive studies should be undertaken to determine the effects of these drugs on the bladder at a cellular and functional level.

Hence, the overall aim of this study was to investigate changes in bladder function as a result of three chemotherapeutic drug treatments, doxorubicin, mitomycin C (MMC) and epirubicin. This will help to identify approaches to reduce the severity of side effects and ultimately improve life quality of the affected bladder cancer patients and also reduce economic burden. It is hypothesised that the three intravesical cytotoxic drugs have effects on bladder function, causing alterations in urothelial mediator release, inflammatory response, urothelial integrity, tissue responses and sensitivity and also efferent nerve responses.

In addition, functions of bladder from young and aged pigs were compared to each other in an attempt to identify age-associated changes in the bladder function.

Aims are addressed in more specific details in the respective experimental chapters.

Chapter 2 - Materials and Methods

Porcine tissue experiments

Materials and equipment were obtained from the following sources.

Chemicals & drugs	Supplier
α,β -methylene ATP Atropine Calcium chloride Carbachol Doxorubicin Glucose Isoprenaline Magnesium sulphate Potassium chloride Potassium dihydrogen phosphate Sodium bicarbonate Sodium chloride	Sigma-Aldrich (St. Louis, USA)
ARL 67156 trisodium salt Epirubicin Mitomycin C N(ω)-Nitro-L-Arginine	Tocris Bioscience (Bristol, UK)
Equipment & softwares	Supplier
Force Transducer LabChart 7 software Octal Bridge Amp PowerLab 8/35	ADInstruments (NSW, Australia)
8 channel tissue bath system Field stimulating electrodes	Radnoti (Monrovia, USA)
S48 Square pulse stimulator	Grass Technologies (Warwick, USA)
Stimulator splitter II	Med-Lab Instruments (Loveland, USA)
Accu-Cut SRM 200 Rotary microtome	Sakura Finetek (Torrance, USA)
Tissue-Tek Dispensing console Tissue-Tek Thermal console	Sakura Finetek (Torrance, USA)
BX41 microscope	Olympus (Victoria, Australia)
Infinity 2 microscopy camera Infinity capture software	Lumenera (Ottawa, Canada)
ImageJ software	Open source
InStat3 software	GraphPad software (La Jolla, USA)
Modulus Micoplate-reader	Turner BioSystems (California, USA)

Fresh bladders from Large White-Landrace pigs were obtained from a local abattoir and immediately placed in cold Krebs-bicarbonate solution (composition, mM: NaCl 118.4, NaHCO₃ 24.9, KCl 4.7, CaCl₂ 1.9, MgSO₄ 1.15, KH₂PO₄ 1.15 and glucose 11.7) at 4°C until use.

Luminal treatment of porcine bladder tissue

The bladders were opened longitudinally with a pair of surgical scissors and sheets of full thickness anterior wall from the dome of the porcine bladder were dissected. Isolated full thickness bladder sheets were set up in modified Ussing chambers (Figure 2.1), where the serosal side was superfused with Krebs-bicarbonate solution (37°C, 5% CO₂ in O₂), whilst doxorubicin, mitomycin C or epirubicin were applied to the urothelial surface at the concentration and duration used clinically. All three cytotoxic drugs were dissolved in Krebs-bicarbonate solution and were allowed to equilibrate for 30 minutes at 37°C immediately before use.

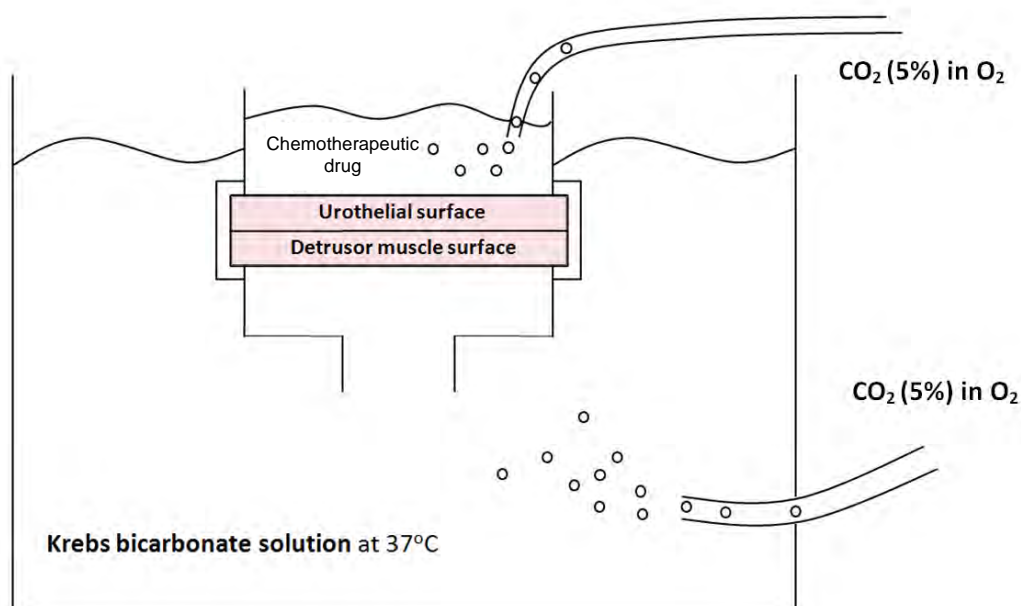


Figure 2.1: Design of double-chamber treatment apparatus which was used to treat the luminal surface of full thickness bladder tissue with doxorubicin, mitomycin C or epirubicin at the clinical concentration and duration of treatment.

Identical incubations with Krebs-bicarbonate solution in the absence of chemotherapeutic drugs were also conducted using matched control bladders (untreated). Following this pretreatment, bladder sheets were washed with Krebs-

bicarbonate solution gently for 3 times to ensure chemotherapeutic agents were removed from urothelial surface and three sets of tissue were isolated for further experiments:

- (i) Full thickness bladder wall with an intact urothelium and lamina propria (will be named “Intact bladder” throughout the thesis)
- (ii) Denuded detrusor muscle strips with the urothelium and lamina propria removed (will be named “Detrusor muscle” throughout the thesis)
- (iii) Strips of urothelium and lamina propria (will be named “Urothelium/LP” throughout the thesis)

Mediator release from pretreated porcine bladder tissue

A common technique that is used to evaluate the involvement of urothelial-derived factors in bladder function is to study the surgically removed “urothelium”. However, since the urothelium is composed of only three to seven layers of cells (Khandelwal et al., 2009), it is difficult, if not impossible, to remove it surgically without including some parts of the underlying lamina propria (Birder, 2010). Hence, our aim was to remove the urothelium and lamina propria (“Urothelium/LP”) together and thereby examine its effects on the detrusor muscle.

Four strips of urothelium/LP (tissues (iii) above) (each strip approx. 5 x 3 mm) were dissected from the pretreated and control bladders before mounting in tissue baths (Radnoti, USA) containing 3ml Krebs-bicarbonate solution warmed at 37°C to assess the effects of chemotherapeutic drugs on basal and stretch-induced mediators from the urothelium/LP. Treated and control urothelial/LP strips were allowed to equilibrate for 1 hour under approximately 20mN tension and were washed with Krebs-bicarbonate solution every 15 minutes. Isometric contractions of urothelial strips were monitored and recorded using a PowerLab data acquisition system (ADInstruments, Australia).

After equilibrium was achieved, 3mL fresh Krebs-bicarbonate solution was added to the tissue baths and the bathing mediums were collected at four different time intervals (1, 2, 3 and 7 min later) for measuring basal ACh and PGE₂ release from the urothelium/LP. The tissue baths were then drained and filled with 3mL fresh Krebs-bicarbonate solution. The bathing mediums were again collected at four different time intervals (1 min to stretch, 2 min to stretch, 1 min after stretch and 5 min after stretch), this time while tissues were stretched over a period of 2 minutes (increasing strip length by 50% of the original length to mimic the physiological change that occurs during filling phase of the bladder) to measure the stretch-induced release of ACh and PGE₂ from the urothelium/LP.

Basal and stretch-induced ATP release from the urothelium/LP were collected by repeating the above steps in the presence of the ecto-ATP inhibitor ARL 67156 trisodium salt (100µM). An ecto-ATP inhibitor was used to minimize the breakdown of ATP into ADP and allow quantification of ATP release from urothelial/LP strips. All the samples were collected on ice and were stored at -80°C for later assay of mediators.

Isolated tissue response from pretreated porcine bladder

Strips of intact bladder (i), detrusor muscle (ii) and urothelium/LP (iii) were dissected from both control and pretreated bladders (each strip approx. 5 x 3 mm). Strips were suspended in tissue baths containing 6ml Krebs-bicarbonate solution under approximately 20mN tension and allowed to equilibrate using the same procedures used for the collection of samples for urothelial mediator release. Isometric contractions of isolated tissue strips were recorded using a PowerLab data acquisition system (ADInstruments, Australia) and saved for analysis.

Contraction to Electrical Field Stimulation (EFS)

Detrusor muscle strips (tissues (ii) above) were stimulated electrically (20V, 0.1ms pulse-width) for 5 seconds every 100 seconds at 1, 5, 10 and 20Hz via silver electrodes placed either side of the tissue. At each stimulation frequency, tissues were allowed to equilibrate and when 3 consistent responses were observed, tissues were exposed to the next stimulation frequency (Figure 2.2). The above steps were repeated in the presence of N(ω)-Nitro-L-Arginine (100 μ M) first, then without washing off drug, contraction to EFS was repeated again in the presence of atropine (1 μ M) and lastly, again without washing off drugs, contraction to EFS was repeated in the presence of α,β -methylene ATP (10 μ M).

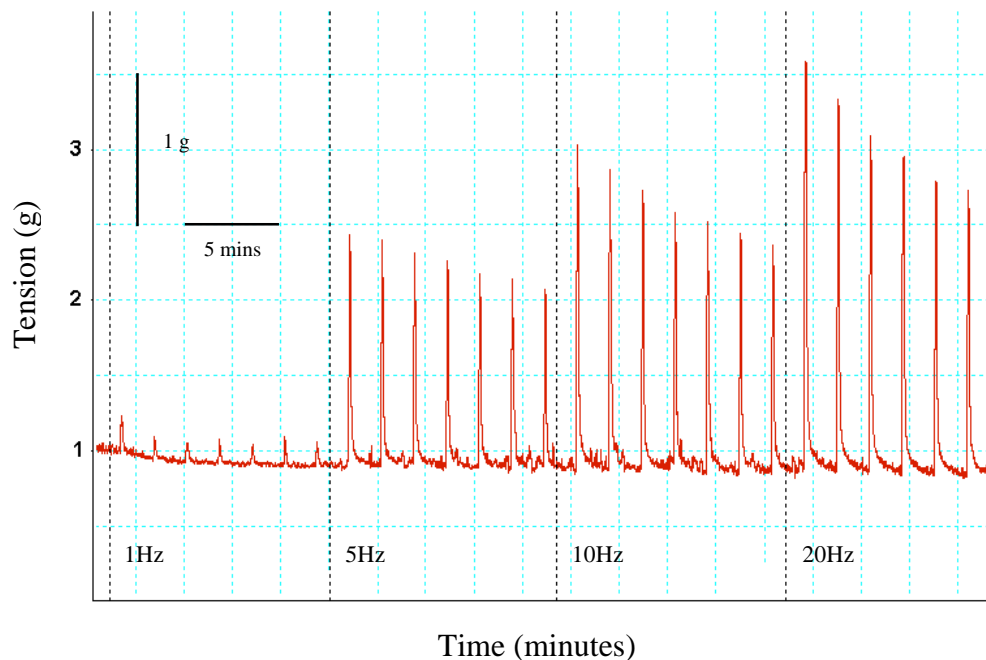


Figure 2.2: Trace demonstrating the responses of detrusor muscle strip to electrical field stimulation (20V, 0.1ms pulse-width, at 1, 5, 10 and 20Hz) delivered as 5 second train every 100 seconds.

Atropine is a non-selective, competitive antagonist of muscarinic acetylcholine receptors (M_{1-5}) (Caulfield and Birdsall, 1998). Alpha, beta-methylene ATP is an agonist that stimulates and then desensitises P2X purinoceptors (Storr et al., 2000). N(ω)-Nitro-L-Arginine is a competitive inhibitor of nitric oxide synthase (NOS) with selectivity for the neuronal and endothelial isoforms of the enzyme (Griffith and Kilbourn, 1996). These were used to investigate if the chemotherapeutic drugs have any influence on the contribution of neurotransmitters NO, ACh and ATP to contractility of the detrusor muscle.

Contraction to Carbachol

To assess the effects of chemotherapeutic drugs on tissue responsiveness, cumulative concentration-response curve to carbachol (up to 273.9 μ M) were obtained on tissues (i), (ii) and (iii) described above (Figure 2.3).

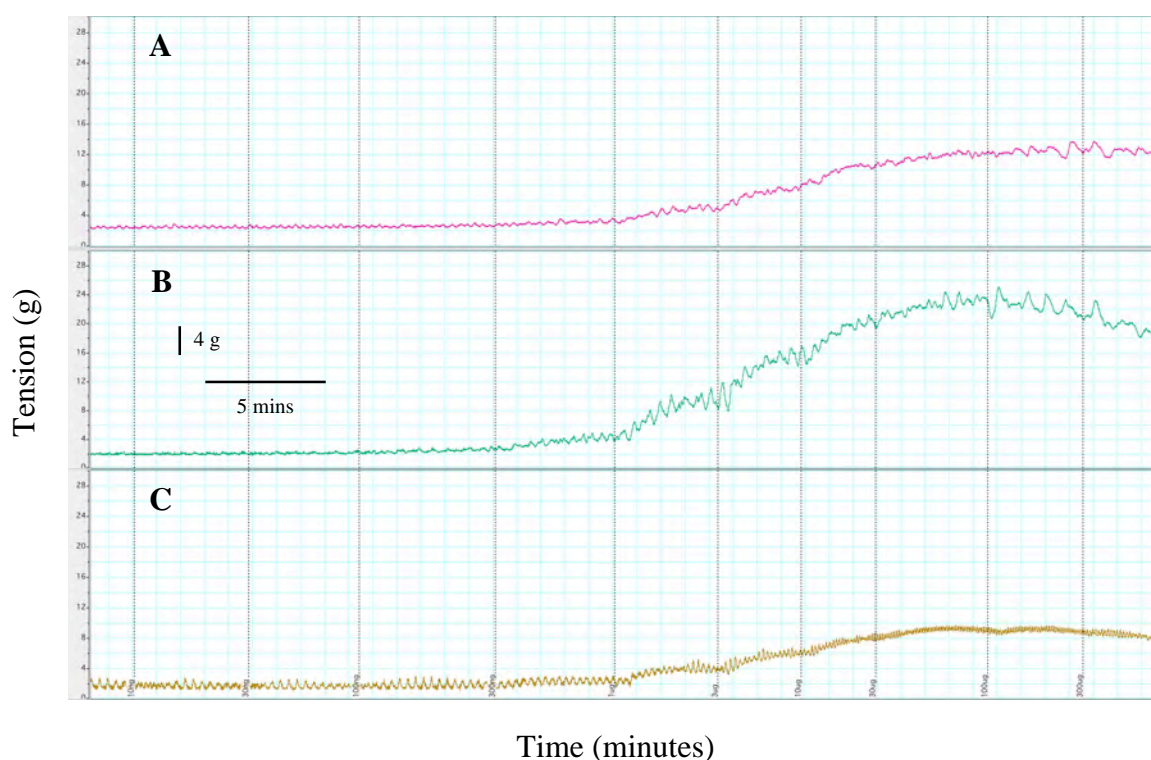


Figure 2.3: Trace demonstrating the cumulative concentration-response curve to carbachol. Strip of intact bladder [A], detrusor muscle [B], and urothelium/LP [C].

Relaxation to Isoprenaline

After the carbachol contraction experiment, the tissues ((i), (ii) and (iii) above) in the organ baths were washed with fresh Krebs-bicarbonate solution every 15 minutes and will be allowed to re-equilibrate back to base tension (20mN) for approximately one and half hours. Then, 27.39 μ M of carbachol was added into each bath to achieve approximately 50 to 80% of maximal response and waited until contractions to plateau. To investigate if chemotherapeutic drugs impact the ability of the tissue to relax, drug stimulated relaxation was observed to cumulative additions of isoprenaline up to 67.3 μ M (Figure 2.4).

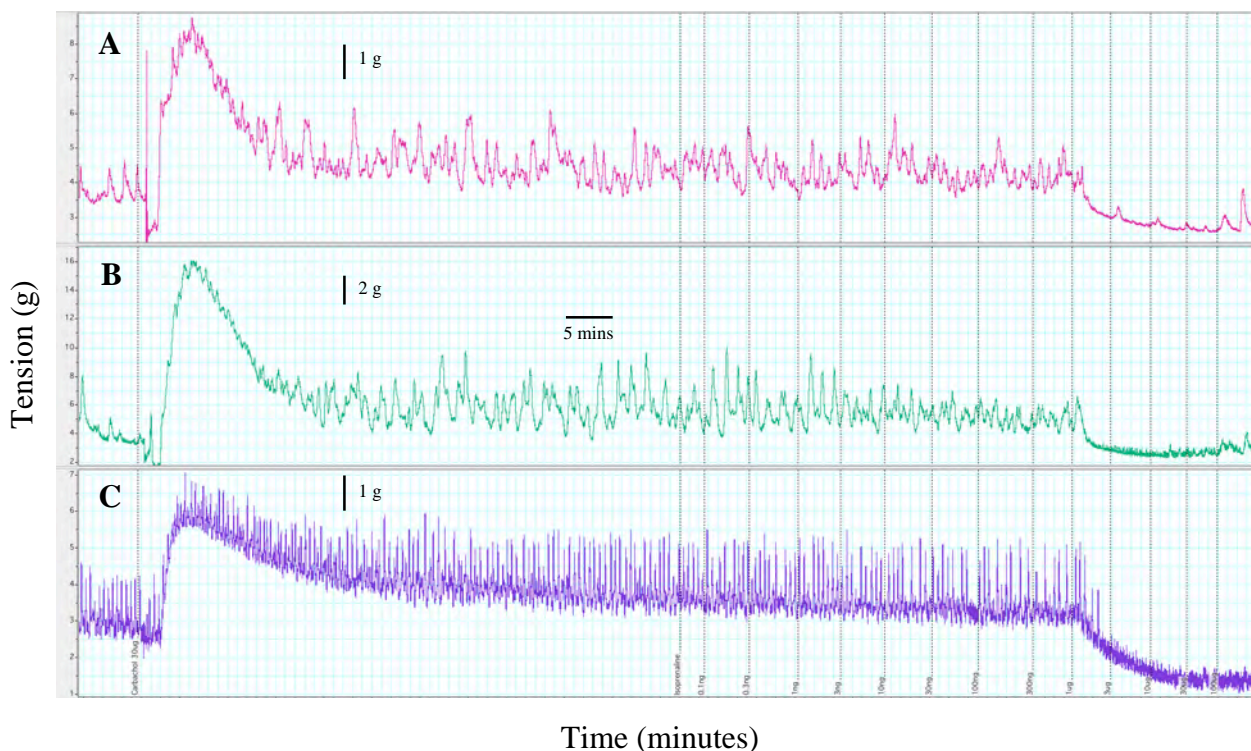


Figure 2.4: Trace demonstrating the cumulative concentration-response curve to isoprenaline. Strip of intact bladder [A], detrusor muscle [B], and urothelium/LP [C].

Microscopic examination of pretreated porcine bladder tissue

Sections of control and pretreated intact bladder dome (tissue (i) above) (each section approx. 5 x 3 mm) were isolated immediately after treatment. These sections were fixed in 4% neutral buffered formalin and left for at least 24 hours at 4°C.

The tissues were then mounted in paraffin wax by placing tissues in solutions as follows:

- (1) 75% ethanol for 1 hour at 4°C.
- (2) 90% ethanol for 1 hour at 4°C.
- (3) 100% ethanol for 1 hour at 4°C.
- (4) 1:1 xylene:ethanol for 30 minutes.
- (5) 100% xylene for 30 minutes.
- (6) Change with fresh 100% xylene for further 30 minutes.
- (7) 1:1 xylene:paraplast bath for 1 hour at 57°C.
- (8) Liquid paraplast for 1 hour at 57°C.
- (9) Change with fresh liquid paraplast for 1 hour at 57°C.

Next, tissues were placed in aluminium rectangle moulds for cross sectioning (cut perpendicular to the surface of the tissue) and completely covered with liquid paraplast and left overnight to set.

Sections were cut at approximately 5µm thickness and placed on uncharged slides and left overnight to dry. Prior to staining with Haematoxylin and Eosin, paraffin on the slides was removed as follows.

- (1) 100% xylene for 1 minute.
- (2) 1:1 ethanol:xylene for 3 minutes.
- (3) 100% ethanol for 3 minutes.
- (4) 90% ethanol for 3 minutes.
- (5) 70% ethanol for 3 minutes.
- (6) Distilled water for 3 minutes.

The sections on the slides were stained as follows. (1) Haematoxylin for 4 minutes. (2) Rinse excess stain with distilled water. (3) Acid alcohol (1ml HCl with 29ml distilled water) for 30 seconds. (4) Rinse with distilled water. (5) Scott's Blue for 30 seconds. (6) Rinse with distiller water. (7) Eosin for 2 minutes. (8) Rinse with distilled water.

The sections were dried overnight and viewed under an Olympus BX41 microscope (Olympus, Australia). Images were taken at 4x, 10x and 40x magnifications using Infinity 2 microscope camera (Lumenera, Canada) attached to a computer using Infinity Capture software (Lumenera, Canada). The urothelial thickness was measured using ImageJ software (open source) and were compared between the treated and control to evaluate the effects of chemotherapeutic drugs on structure of the urothelium. At least 10 urothelial thickness measurements were obtained from each bladder section, with 4 bladders examined from each group.

Data and statistical analysis

At the end of each experiment, the weights of tissues were recorded for normalising data. Mean (\pm SEM) increases in tension induced by carbachol or electrical field stimulation were calculated. For response to carbachol, mean (\pm SEM) $-\log EC_{50}$ (pEC_{50}) values and maximum responses were calculated from the best-fit concentration-response curves by use of GraphPad PRISM software. Mean (\pm SEM) percentage decreases in pre-contraction to carbachol induced by isoprenaline were calculated. Mean (\pm SEM) $-\log IC_{50}$ (pIC_{50}) values and minimum responses were calculated from the best-fit concentration-response curves. Similarly for the mediator release study, mean (\pm SEM) concentrations were determined before and after stretch and data for treated and control bladders were compared. Data were analysed using a paired Student t-test or one-way ANOVA with Dunnett multiple comparisons test, using GraphPad InStat3 software. Significance levels were defined as $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***)

Cell culture experiments

Materials and equipment were obtained from the following sources.

Chemicals & drugs	Supplier
Foetal bovine serum D-Glucose L-Glutamine McCoy's 5A culture medium Phosphate buffered saline Penicillin/Streptomycin Trypan Blue dye Trypsin-EDTA	Invitrogen (Victoria, Australia)
Calcium chloride DMSO Dulbecco's modified eagle's medium Glucose HEPES sodium salt Magnesium chloride Potassium chloride Sodium chloride	Sigma-Aldrich (St. Louis, USA)
Epirubicin Mitomycin C	Tocris Bioscience (Bristol, UK)
Bovine serum albumin	Santa Cruz Biotechnology (Dallas, USA)
Equipment & consumables	Supplier
24 well plate 25 cm ² tissue culture flask 75cm ² tissue culture flask Sterile pipettes	TPP (Trasadingen, Switzerland)
Countess automated cell counter	Invitrogen (Victoria, Australia)
Modulus Micoplate-reader	Turner BioSystems (California, USA)
BD FACSV TM erse	BD Biosciences (NSW, Australia)

Two human urothelial cell lines (RT4 and UROtsa) were used to assess the cell specific effects of MMC and epirubicin on urothelial function. Specific details relating to choice of cells, routine culture and treatment are provided in Chapter 5 and 6.

Resazurin reduction assay - cell viability

Immediately, 24 hour and 1 week after drug treatment and also 1 week after second treatment, reduction of the redox dye resazurin to resorufin was used to measure the viability of cell cultures. Viable cells can be monitored by using resazurin which is a cell permeable redox indicator. Resazurin (blue and non-fluorescent) can be reduced by viable cells with active metabolism into resorufin which is pink and fluorescent. The amount of resorufin produced is directly proportional to the number of viable cells which can be quantified using a microplate fluorometer (Riss et al., 2004). Cell specific details provided in Chapter 5 and 6.

Data and statistical analysis

Data for ACh, ATP and PGE₂ response to hypotonic stimulation were calculated by subtracting the concentration of basal release from the concentration of hypo-osmotically stimulated release. Results are expressed as mean (\pm SEM). Data were analysed using a paired Student's t-test or one-way ANOVA with Dunnett multiple comparisons test, using GraphPad InStat3 software. Significance levels were defined as $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***).

Assays for urothelial mediators, NO and inflammatory cytokines

The urothelial mediators (ACh, ATP, and PGE₂), NO and inflammatory cytokines in samples collected from each experiment were measured using commercially available kits. Throughout the thesis, the measured concentration of these factors in samples, which is affected by breakdown and reuptake mechanisms, will be referred to as 'release' (e.g., decreased ACh release in control cells, and increased release of ATP in tissues pretreated with cytotoxic drugs).

Acetylcholine

ACh was measured using Amplex® Red ACh Assay kit purchased from Molecular Probes. This is a fluorescence based assay that relies on acetylcholinesterase (AChE) converting ACh (present in samples and standards) into choline. Choline is then oxidised by choline oxidase to betaine and H₂O₂. In the presence of horseradish peroxidase, H₂O₂ reacts with Amplex Red reagent to generate the highly fluorescent product resorufin. Thus, this assay measures both ACh and hydrolysed products of ACh.

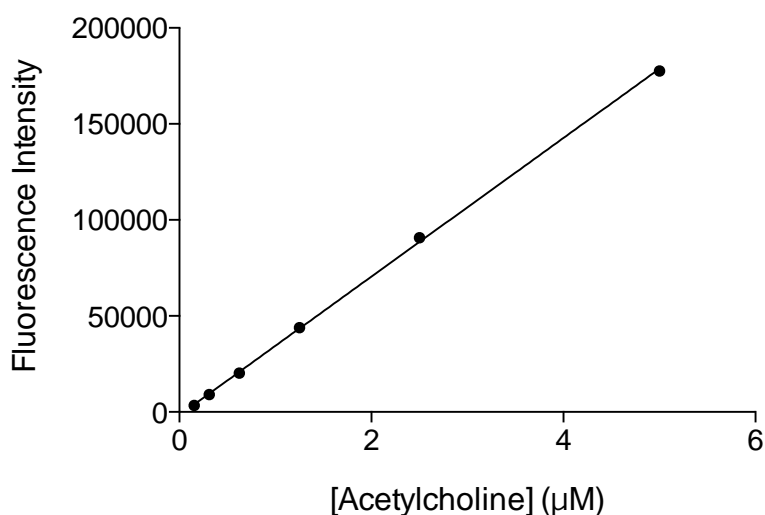


Figure 2.5: Representative acetylcholine (ACh) standard curve. $R^2 = 0.9997$.

First, vial of Amplex Red Reagent was dissolved into 200μl DMSO. A 1X working solution of Reaction Buffer was prepared by adding 5ml of 5X Reaction Buffer

solution to 20ml of distilled water (dH₂O). A 100mM solution of ACh was prepared by dissolving 5mg of acetylcholine chloride in 275µl of dH₂O. ACh standard curve was prepared by diluting 100mM acetylcholine stock solution into 1X Reaction Buffer to produce ACh concentrations of 0 to 5µM. 100µl of the sample or standard were added into each well. A working solution of 400µM Amplex Red reagent containing 2U/ml HRP, 0.2U/ml choline oxidase and 1U/ml AChE by adding 200µl of Amplex Red reagent stock solution and 100µl of the HRP stock solution, 100µl of choline oxidase stock solution and 100µl of the AChE stock solution to 9.4ml of 1X Reaction Buffer. This 10ml volume is sufficient for ~100 assays. 100µl of working solution was added into each well and incubated for 30 minutes at 25°C protected from light. Fluorescence was measured on a Modulus Microplate reader (Ex. 540 / Em. 590nm). The ACh concentration in samples was calculated using standard curves constructed from known ACh standards (Molecular Probes, 2004) (Figure 2.5). ACh concentrations measured were normalised to weight of strips measured at the end of porcine tissue experiments, or normalised to controls using corresponding resazurin reduction data for cell culture experiments.

Adenosine triphosphate

ATP release was measured using ATP Determination kits from Molecular Probes. This is a bioluminescence assay for determination of ATP using luciferase and its substrate D-luciferin. The assay was performed according to manufacturer's protocol.

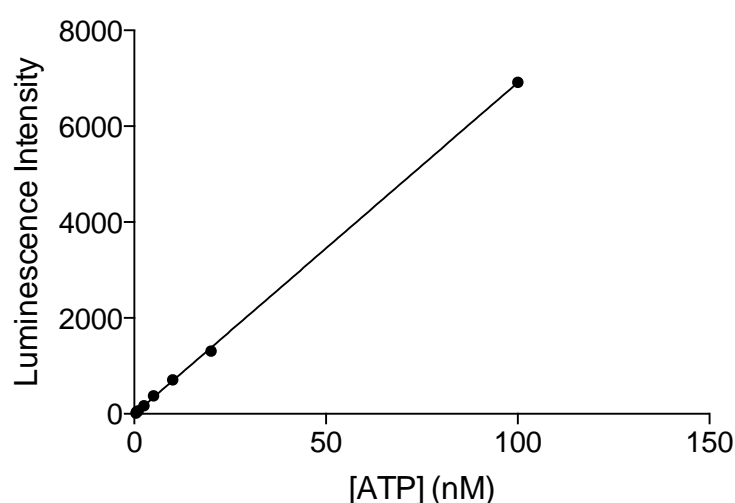


Figure 2.6: Representative ATP standard curve. $R^2 = 0.9998$.

First, 1.0ml of 1X Reaction Buffer was prepared by adding 50 μ l of 20X Reaction Buffer to 950 μ l of distilled water (dH₂O). This 1.0ml of 1X Reaction Buffer was added to a vial of D-luciferin to make a 10mM D-luciferin stock solution and this solution was protected from light until use. ATP standard curve was prepared by diluting 5mM ATP stock solution into dH₂O (or solution containing 100 μ M ARL67156 trisodium salt) to produce ATP concentrations of 0 to 500nM. 10 μ l of samples or standards were added into each well. A reaction solution was prepared by adding 0.5ml of 20X Reaction Buffer, 0.1ml of 0.1M DTT, 0.5ml of 10mM D-luciferin and 2.5 μ l of firefly luciferase (5mg/ml stock solution) to 8.9ml of dH₂O. This 10ml volume is sufficient for ~100 assays. 100 μ l of reaction solution was added into each well and incubated for 15 minutes protected from light. Luminescence was measured on a Modulus microplate reader. A standard curve using known concentrations of ATP was constructed and used to calculate ATP concentration in samples (Molecular Probes, 2006) (Figure 2.6). ATP concentrations measured were normalised to weight of strips measured at the end of porcine tissue experiments, or normalised to controls using corresponding resazurin reduction data for cell culture experiments.

Nitric oxide

NO was measured using Nitrate/Nitrite Fluorometric Assay kit provided by Cayman Chemical Company. This kit provides an accurate and convenient method for measurement of total nitrate/nitrite concentration. The assay works by adding nitrate reductase to samples/standards to convert nitrate to nitrite. The second step is the addition of DAN reagent which results in the production of a fluorescent product, 1 (H)-naphthotriazole. The fluorescence measurement of this compound accurately determines nitrite concentration in the sample.

The contents of the Assay Buffer vial were diluted to 100ml with distilled water (dH₂O). The contents of the Nitrate Standard vial were reconstituted with 1.0ml of Assay Buffer. 0.1ml of reconstituted Nitrate Standard was added to 0.9ml of Assay Buffer to make stock standard. A nitrate standard curve was prepared by diluting 200 μ M nitrate stock solution into Assay Buffer to produce nitrate concentrations of 0 to 500pmoles. 80 μ l of Assay Buffer (or culture medium when applicable) was added to the blank wells and 30 μ l to standard wells. 50 μ l of each Nitrate standard tubes

were added to the dedicated wells. 10µl of samples were added to the sample wells and the volume was adjusted to 80µl with Assay Buffer. 10µl of the Enzyme Cofactor Mixture was added to each well. 10µl of the Nitrate Reductase Mixture was added to each well. The plate was covered and incubated at room temperature for 30 minutes (or 1 hour when assaying tissue culture medium). After the required incubation time, 10µl of DAN reagent was added to each well and incubated for 10 minutes. 20µl of NaOH was added to each well. Fluorescence was measured using a Modulus microplate reader (Ex. 360nm / Em. 430nm). A standard curve using known concentrations of nitrate was constructed (fluorescence vs. picomoles) (Figure 2.7).

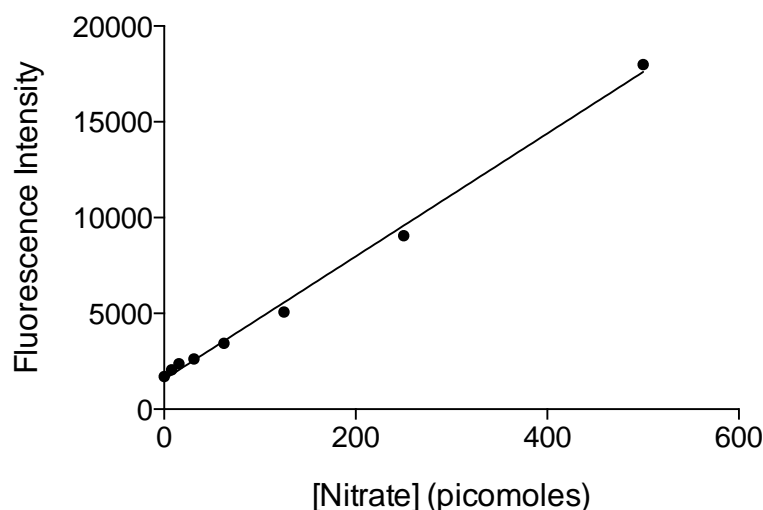


Figure 2.7: Representative trace of nitrate and nitrite standard curve. $R^2 = 0.996$.

Sample nitrate concentrations were determined by following equation.

$$[\text{Nitrate+Nitrite}] (\mu\text{M}) = \left(\frac{\text{fluorescence} - y \text{ intercept}}{\text{slope}} \right) \left(\frac{1}{\text{volume of sample used } (\mu\text{l})} \right) \times \text{dilution}$$

Where dilution is a sample dilution done prior to addition of the sample to the plate (Cayman Chemical Company, 2011). Metabolites of NO (nitrate and nitrite) concentrations measured were normalised to weight of strips measured at the end of porcine tissue experiments, or normalised to controls using corresponding resazurin reduction data for cell culture experiments.

Prostaglandin E₂

The level of PGE₂ released was measured spectrophotometrically using the Prostaglandin E₂ EIA Kit - Monoclonal provided by Cayman Chemical Company. This kit is a competitive assay that can be used for quantification of PGE₂. The competition between PGE₂ and a PGE₂-AChE conjugate (PGE₂ Tracer) for a limited amount of PGE₂ monoclonal antibody is the basis of this assay.

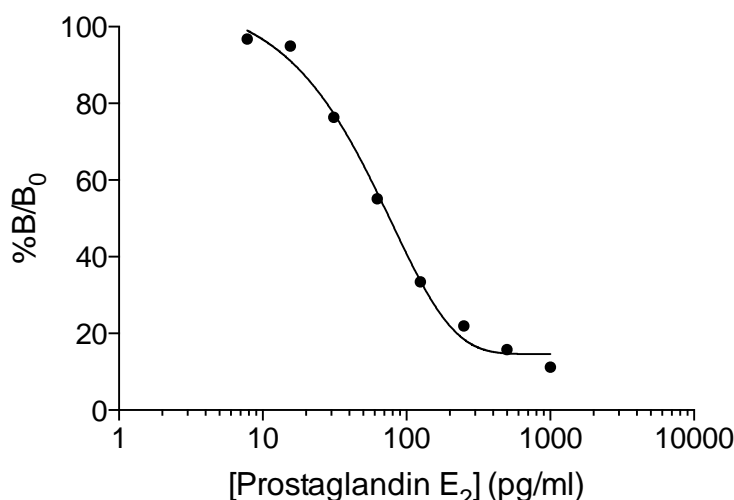


Figure 2.8: Representative prostaglandin E₂ standard curve. %B/B₀ is standard bound/Maximum bound. $R^2 = 0.9945$.

The contents of one vial of EIA Buffer Concentrate (10ml of 10X) were diluted with 90ml of dH₂O. The contents of the PGE₂ EIA Standard were reconstituted with 1.0ml of EIA Buffer to make stock standard solution. A PGE₂ standard curve was prepared by diluting 10ng/ml of PGE₂ stock solution into EIA Buffer to produce PGE₂ concentrations of 0 to 1000pg/ml. 100μl of EIA Buffer was added to non-specific binding (NSB) wells. 50μl of EIA Buffer was added to maximum binding (B₀) wells. 50μl of standards were added to each standard wells. 50μl of sample per well was added. 50μl of PGE₂ AChE Tracer was added to each well except the total activity (TA) and the blank (Blk) wells. 50μl of PGE₂ Monoclonal Antibody was added to each well except the TA, the NSB and the Blk wells. The plate was covered with plastic film and incubated for 18 hours at 4°C. After the required incubation time, the wells were emptied and rinsed five times with Wash Buffer. 200μl of Ellman's Reagent was added to each well. 5μl of PGE₂ AChE Tracer was added to the TA wells. The plate was covered with plastic film and the plate was developed by using

orbital shaker with a cover (protect from light) for 60 to 90 minutes. Samples and standards were analysed and the absorbance measured on Modulus Microplate reader (420nm) was directly proportional to the [bound tracer] and inversely proportional to the [PGE₂] (Cayman Chemical Company, 2010) (Figure 2.8). PGE₂ concentrations measured were normalised to weight of strips measured at the end of porcine tissue experiments, or normalised to controls using corresponding resazurin reduction data for cell culture experiments.

Inflammatory cytokine

Cytokines (IL-8, IL-1 β , IL-6, IL-10, TNF and IL-12p70) were measured using a BD[™] Cytometric Bead Array Human Inflammatory Cytokine Kit according to the manufacturer's instructions. In this kit, six bead populations with distinct fluorescence intensities are coated with capture antibodies specific for IL-8, IL-1 β , IL-6, IL-10, TNF, and IL-12p70 proteins. During the assay procedure, the inflammatory cytokine capture beads are mixed with the recombinant standards or unknown samples and are incubated with the PE-conjugated detection antibodies to form sandwich complexes. The intensity of PE fluorescence of each sandwich complex reveals the concentration of that cytokine.

First, the number of assay tubes which includes samples, standards and controls required for the experiment are determined. For each assay tube to be analysed, a 5 μ l aliquot of each capture bead suspension were added into a single tube labelled 'Mixed Capture Beads'. The contents of one vial of lyophilised Human Inflammatory Cytokine Standards were reconstituted with 2.0ml of assay diluent. This is the highest standard concentration. A standard curve was prepared by diluting this standard into assay diluent to produce Human Inflammatory Cytokine concentrations of 0 to 5,000pg/ml. The mixed capture beads that were prepared previously were vortexed and 25 μ l was added to all assay tubes. 25 μ l of the standards were added to the control assay tubes. 25 μ l of each unknown sample was added to the appropriately labelled sample assay tubes. 25 μ l of the Human Inflammatory Cytokine PE Detection Reagent was added to all assay tubes. The assay tubes were incubated for 3 hours at room temperature, protected from light. Following incubation, 500 μ l of wash buffer was added to each assay tubes and centrifuged at 200g for 5 minutes. The supernatant was carefully aspirated and

discarded from each assay tube. 300µl of wash buffer was added to each assay tube to resuspend the bead pellet. Standards and samples were acquired on a BD FACSVerse[™] flow cytometer to measure fluorescence (BD Biosciences, 2010). Concentrations of inflammatory cytokines were normalised to controls using corresponding resazurin reduction data.

Chapter 3 - The effects of doxorubicin on young and aged porcine bladder tissue

Introduction

Efficacy and adverse effects of intravesical doxorubicin

A previous study by Garnick et al. (1984) has evaluated the effects of intravesical doxorubicin therapy on disease recurrence and bladder toxicity for patients with multiple recurrent superficial transitional cell carcinoma of the bladder. Doxorubicin was administered intravesically in 27 patients for 60 minutes, with the starting concentration of 60mg diluted in 40 to 50ml saline solution and concentrations were increased to 90mg. Treatments were performed every 3 weeks for a total of 8 doses, then every 6 weeks for 2 doses and then every 12 weeks for 2 doses. Therapy ended for patients once rendered free of disease. Cystoscopy and urinary cytology analysis revealed that of the patients 30% experienced intermittent episodes of dysuria, 26% reported urinary frequency, 41% had hematuria and 15% had bladder spasms. However, none of these toxicities led to discontinuation of the drug. The study also found that 56% of the patients maintained complete eradication of bladder cancer while 33% developed recurrent disease while on therapy. Cystoscopy has remained grossly negative in 3 (11%) patients who have had positive class 5 (conclusive for malignancy) cytological analysis.

Lamm et al. (1991) also evaluated efficacy and side effects of intravesical doxorubicin chemotherapy. A total of 135 eligible patients with rapidly recurrent (stage Ta or T1) or in situ transitional cell carcinoma of the bladder were administered intravesically with 50mg of doxorubicin diluted in 50ml saline. The initial treatment was given within 3 days after transurethral resection of bladder tumour (TURBT) (the solution was retained for 30 minutes), followed by 4 weekly treatments and then by 11 monthly treatments. The solution was retained for 2 hours for subsequent treatments. In patients with carcinoma in situ, the probability of being disease free at 5 years was 17% after intravesical doxorubicin. The median times to treatment termination due to persistence (treatment failure), recurrence or progression of disease was 10.4 months. In patients with carcinoma in situ, the estimated probability of documented disappearance of disease was 34%, the median times to treatment failure was 5.1 months, and the probability of being disease free at 5 years was 18%. The study also reported local adverse effects which included 26.7% and 48.9% of the patients experiencing haematuria and irritative bladder symptoms (dysuria, increased frequency, or urgency), respectively. Systemic toxicities such as

nausea, vomiting or anorexia in 8.9%, fever or chills in 8.1%, malaise in 12.6% and diarrhea in 1.5%, of the patients were observed.

The efficacy and side effects of intravesical doxorubicin in superficial bladder cancer following TURBT was assessed by Huang et al. (2003). Thirty patients with transitional cell carcinoma received 30mg doxorubicin in 50ml saline for 2 hours. The instillation was performed every week during first month followed by monthly instillation for 11 times. The recurrence rate was 30% and the median recurrence-free survival was 20 months. The bladder toxicities dysuria, urinary frequency, haematuria, urinary infection and bladder spasm occurred in 13.3%, 16.7%, 13.3%, 10% and 6.7% of the patients.

A more recent study by Al-Gallab et al. (2009) incorporated 85 patients with non-muscle invasive bladder cancer Ta (27%) and T1 (73%) who were treated intravesically with 50mg doxorubicin in 50ml saline for 2 hours. First session started a week after TURBT and repeated weekly 4 times then monthly 11 times. The study found that the total tumour recurrence rate was 23.5%. Of these, tumour recurred in 35% of patients with Ta and in 65% of patients with T1. Also, it was demonstrated that 16.5%, 14.1%, 14.1%, and 9.4% of the patients developed urinary frequency, dysuria, haematuria, and urinary tract infection, respectively.

***In vitro* model, Porcine Bladder**

In this study, porcine bladder tissue was used as an *in vitro* model to investigate the effects of doxorubicin treatment on normal bladder function. In scientific research, pig was one of the earliest animals used and this was due to the remarkable similarities between pigs and humans. The anatomical, physiological, immunological, metabolic and nutritional similarities with human makes pig a unique and viable model for studying many human diseases (Verma et al., 2011). In the field of bladder research, porcine bladder is also used widely both *in vivo* and *in vitro* as they are known to closely resemble the human bladder (Akino et al., 2008; Arentsen et al., 2011; Bramich and Brading, 1996; Bridgewater et al., 1993; Mills et al., 2000; Moro et al., 2012; Murakami et al., 2007).

It has been demonstrated in various studies that the composition of bladder wall is similar between human and pig in which both consist of urothelium (~0.1 mm),

lamina propria (~0.3 mm), and the detrusor smooth muscle divided into an inner and an outer tunica muscularis (~3 mm total thickness) (Dixon and Gosling, 1983; Narumi et al., 1993; Teufl et al., 1997). Thus, pig and human bladder are structurally similar but this also extends to bladder physiology and pharmacology. The subtype of β -adrenoceptor in the detrusor smooth muscle that mediates relaxation appears to be species-dependant, but it has been reported that pigs have a similar population of β -adrenoceptor subtypes to human with β_3 -adrenoceptor predominating and a minor population of β_2 -adrenoceptors (Yamanishi et al., 2002b). The muscarinic M_2 to M_3 receptor ratio in porcine bladder is similar to that in human (both 3:1) (Wang et al., 1995) and also the muscarinic subtype that leads to bladder contraction is mediated via the minor population of M_3 muscarinic receptor in both pig (Sellers et al., 2000) and human bladder (Chess-Williams et al., 2001). In addition, a study by Kumar et al. (2004) has demonstrated that the quantity of ATP release, the proportion of ATP release from neuronal and non-neuronal sources, and the activity of ecto-ATPases affecting ATP levels in response to mechanical stretch and electrical field stimulation are similar in pig and human bladder.

Despite many advantages of using porcine bladder in scientific research, there are also limitations of this model. A large animal model like pig is more expensive and difficult to manage compared to rodents. However, in the long-term, they often prove to be cost-effective in providing more reliable and worth-while results because of their excellent physiological similarity to humans (Doyle et al., 1968; Verma et al., 2011). Another disadvantage when using tissues isolated from animal models is that they can only be kept for a short period of time without deterioration of function. For example, the life span of the detrusor muscle and the urothelium are limited to approximately 2 days and 1 day, respectively. This makes long-term studies difficult and recovery studies impossible. Furthermore, it has been reported that the immune response differs slightly from one pig to another, and also sex and age have been reported to affect the protein profile (Rothkotter et al., 2002), although this may simply reflect what also occurs in humans.

The porcine bladders obtained from the local abattoir have been previously used in my Honours study to assess the effect of doxorubicin treatment on normal bladder function. Despite the fact that bladder cancer is primarily a disease of the elderly, with incidence peaking at 85 years of age (Schultzel et al., 2008), the bladders from

young pigs (4 to 6 months) were used in these experiments because it was more frequently available compared to tissues from older pigs (1 to 2 years; these tissues will be called “aged” throughout the thesis) in addition to the constrained timeframe for the Honours research.

Effects of Ageing on bladder function

In almost every physiological system, ageing is associated with declining function. The function of the gastrointestinal tract is altered as you age and some changes include impaired acid secretion and, decreased absorptive surface, splanchnic blood flow and gastrointestinal motility (Geokas and Haverback, 1969; Greenblatt et al., 1982). A study by Wynne et al. (1989) has reported that between the ages of 20 and 80 years, liver size decreases by 18 to 44% and hepatic blood flow declines at a rate of 0.3 to 1.5% per year after the age of 25. Also, decline in renal weight, renal blood flow, the number of glomeruli and the glomerular filtration rate occurs in a linear fashion after the age of 30 years (Anderson and Brenner, 1986; Brown et al., 1986). In addition, it is well established that lung function declines with age such as decrease in the vital capacity and forced expiratory volume, and reduced elastic force of the lung (Knudson, 1991). Ageing is also associated with deteriorated function of the bladder.

Changes in the morphology of the bladder in response to ageing has been reported by Gosling (1997). The aged bladders demonstrated a normal morphology of smooth muscles cells packed closely together with relatively little intervening connective tissue, but a reduction in the innervation of the smooth muscle cells (autonomic presumptive cholinergic nerve) was observed compared to normal bladders.

In a report by Chun et al. (1988), a 52% and 100% increase in frequency of micturition and voiding pressure, respectively, were demonstrated in aged male rats (22 to 24 months old) compared to young male rats (5 to 7 months old).

A previous study by Saito et al. (1999) compared blood flow to the bladder and detrusor function in young (6 months old) and aged (24 months old) male rats and reported various age-related changes. The study found that in both young and aged rats, blood flow to the bladder decreased as intravesical volume increased and was smaller in aged rats compared to young rats. Also, voiding pressure was smaller and

bladder capacity was larger in aged rats. Impairment in the pressure increase, in response to bethanechol and to low frequency field stimulation was observed in aged rats. In addition, compliance of isolated bladders of aged rats was greater and peak response to field stimulation was observed at a larger capacity. These findings suggest that the changes in bladder function with ageing may correlate with a decrease in blood flow to the bladder.

Lluel et al. (2000) also reported changes in the bladder function of female rats with ageing. While 60% of conscious aged rats (30 months old) showed spontaneous contractions during the bladder-filling phase, only 25% of young adult rats (10 months old) showed spontaneous contractions. Micturition pressure and duration of micturition were significantly higher by approximately 40% to 50% in aged rats. Contrarily, bladder capacity, bladder compliance, micturition volume, and residual volume were not changed with age. In addition, *in vitro* studies have revealed that while contractile responses of the bladder body to KCl, carbachol, arecoline, and α,β -mATP were similar in tissues from young and aged rats, maximum responses to noradrenaline were increased by two-fold in the aged rats. The potency of isoprenaline in relaxing KCl-precontracted bladder body was similar in both age groups. Furthermore, a significant increase in the mean thickness of the muscularis layer with age was observed, while the collagen density significantly decreased in the muscularis and in the lamina propria layers with age.

A recent study using male rats has also demonstrated significant age related differences in bladder function. An increase in bladder capacity, post-void residual volume, micturition volume and frequency, baseline and intermicturition pressure, and spontaneous activity, and a decrease in micturition pressure were observed in aged rats (28 to 30 months old) compared to young rats (4 to 6 months old). The study also reported significantly lower responses of bladder strips to carbachol and electrical field stimulation in aged rats and histological examination revealed urothelial thinning, lower muscle mass and higher collagen contents in the bladders of aged rats compared to young rats (Zhao et al., 2010).

Alterations in bladder function have been also observed with ageing in human. An urodynamic study of 436 patients (253 men and 183 women, 40 years of age or older) has shown that, in both sexes, postvoid residual urine volume increased, and peak flow rate, average flow rate, voided volume and bladder capacity decreased with age

(Madersbacher et al., 1998). Hence, it raises concern that elderly patients with reduced function of organs may develop severe side effects after chemotherapeutic treatment. There is a study supporting this view which reported an increased incidence of side effects in elderly cancer patients treated with chemotherapy compared to younger subjects (Goto et al., 2012).

Aims

Despite the large number of patients receiving doxorubicin intravesically and the high percentage of those suffering urological adverse effects, there have been no investigations of the actions of this agent on the non-malignant tissues of the bladder.

Thus, the aim of the present study was to investigate the effects of doxorubicin treatment on (1) basal and stretch-induced release of mediators (ACh, ATP and PGE₂) from strips of urothelium/LP, (2) tissue responsiveness and sensitivity of bladder tissues (intact bladder, detrusor muscle and urothelium/LP) to cholinergic and adrenergic stimulation, (3) neurogenic response of detrusor muscle strips to electrical field stimulation, and (4) integrity of the urothelium, LP and detrusor muscle. These studies aimed to determine whether changes in the local bladder mechanisms may provide an explanation for the bladder dysfunction noted by patients following intravesical treatment with doxorubicin. Experiments were performed on both young and aged porcine bladders to also identify the effects of ageing on the bladder function and response to doxorubicin treatment.

Some of the experiments on young pigs in the following chapter were included in my Honours thesis. These included contractile responses of detrusor muscle strips to carbachol and mediator release from urothelial/LP strips. However, the experiments were repeated during my PhD project to provide contemporary controls to compare with the aged animals and to increase N values, thus allowing a more accurate comparison of tissues from young and aged pigs.

Materials and Methods

(Refer to “Porcine tissue experiments” and “Assays for urothelial mediators, NO and inflammatory cytokines” sections of Chapter 2 for full details)

Fresh bladders from two groups of pigs were used in this study:

- (1) Young pigs (4 to 6 months old; approx. 80kg)
- (2) Aged pigs (2 to 3 years old; approx. 250kg)

Throughout the thesis, bladders from young pigs will be named “Young bladders” and bladders from aged pigs will be named “Aged bladders”.

A clinically used concentration (1mg/ml diluted in fresh Krebs-bicarbonate solution) of intravesical doxorubicin was applied to the luminal surface of full thickness sheets of bladder wall by using double-chamber treatment apparatus for 1 hour (clinical duration).

Following this pretreatment, strips of intact bladder, detrusor muscle and the urothelium/LP were isolated to assess the effects of doxorubicin on tissue responses, and mediator release, and whole bladder sections were also isolated to evaluate the bladder morphology after treatment. The release of mediators (ATP, ACh, and PGE₂) from the urothelium/LP was measured using commercially available kits.

Results

Contractile responses to carbachol

Influence of age on responses of untreated (control) tissues

All tissue strips (intact bladder, detrusor muscle and urothelium/LP) from control incubated young and aged bladders contracted to carbachol (Figure 3.1).

In both young and aged control tissues, when expressed as the force developed per gram of tissue, the largest contractions to carbachol were produced by the urothelium/LP tissues, which produced responses significantly ($P < 0.01$) greater than the detrusor muscle tissues, which in turn produced contractions significantly (young $P < 0.01$; aged $P < 0.001$) greater than the intact bladder tissues (Table 3.1).

The potency (pEC_{50} values) of carbachol was similar on detrusor muscle tissues from both young and aged control bladders but the potency was higher in intact bladder and urothelial/LP tissues from young control bladders compared to aged control bladders (Table 3.1).

The maximum responses to carbachol on all tissues types were significantly greater (5-fold) in control tissues from young bladders compared to control tissues from aged bladders. Thus, with increasing age, there is reduction in the contractions produced by the various tissue types within the bladder.

Effect of doxorubicin pretreatment (Young & Aged)

All tissue strips from doxorubicin pretreated young and aged bladders contracted to carbachol (Figure 3.1) and doxorubicin pretreatment did not affect the potency (pEC_{50} values) of carbachol on any of the tissues from either young or aged bladders (Table 3.1).

Pretreatment with doxorubicin (1mg/ml) for 1 hour did not alter subsequent responses of the detrusor muscle tissues or intact bladder tissues from young bladders to carbachol (Figure 3.1; Table 3.1). However, doxorubicin pretreatment did enhance subsequent responses of the urothelium/LP tissues from young bladders to carbachol (Figure 3.1).

In contrast, in all three tissue types of aged bladders, doxorubicin pretreatment significantly enhanced subsequent responses to carbachol (Figure 3.1; Table 3.1).

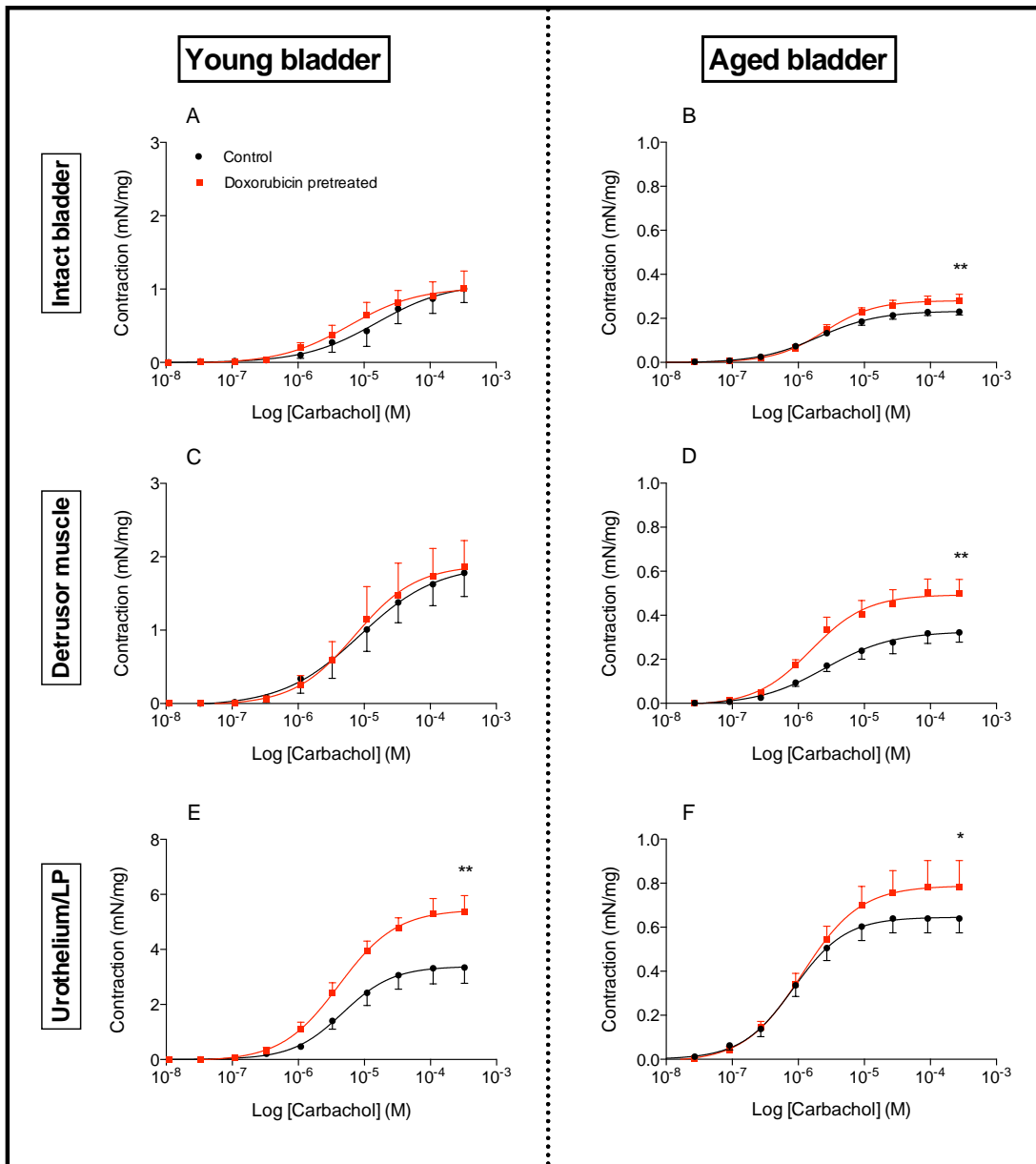


Figure 3.1: Cumulative concentration-response curves to carbachol of tissues from control and doxorubicin pretreated bladders from young and aged animals. Responses of intact bladder [A & B], detrusor muscle [C & D] and urothelium/LP [E & F]. Data are expressed as means \pm SEM. * $P < 0.05$ and ** $P < 0.01$ compared to maximum response of control tissue. Note differences in scales between tissues from young and aged bladders.

Table 3.1: Potency (pEC₅₀) values and maximum responses (mN/mg tissue) to carbachol of intact bladder strips, detrusor muscle strips and strips of urothelium/LP from the bladders of young and aged animals. Data are expressed as means \pm SEM.

Young bladders						
Tissue	Control (untreated) bladders			Doxorubicin-pretreated bladders		
	Potency	Maximum	N	Potency	Maximum	N
Intact bladder	4.81 \pm 0.35	1.08 \pm 0.25	6	5.23 \pm 0.26	1.02 \pm 0.15	5
Detrusor muscle	5.07 \pm 0.29	1.89 \pm 0.34	6	5.14 \pm 0.26	1.88 \pm 0.29	6
Urothelium/LP	5.33 \pm 0.14	3.39 \pm 0.27	4	5.39 \pm 0.09	5.45 \pm 0.28	5
Aged bladders						
Intact bladder	5.69 \pm 0.08 ^a	0.23 \pm 0.01 ^b	6	5.61 \pm 0.09	0.28 \pm 0.01	6
Detrusor muscle	5.59 \pm 0.17	0.33 \pm 0.03 ^b	6	5.81 \pm 0.14	0.49 \pm 0.03	6
Urothelium/LP	6.07 \pm 0.11 ^b	0.65 \pm 0.03 ^c	6	5.94 \pm 0.14	0.79 \pm 0.05	6

N number of bladders examined

^a P<0.05 compared to young control tissue

^b P<0.01 compared to young control tissue

^c P<0.001 compared to young control tissue

Relaxation responses to isoprenaline

Influence of age on responses of untreated (control) tissues

All tissues from the control young and aged bladders relaxed to isoprenaline (Figure 3.2). The potency (pEC_{50} values) of isoprenaline was similar on intact bladder tissues and detrusor muscle tissues. However, the potency was significantly ($P<0.01$) higher in control urothelial/LP tissues from young bladders compared to aged bladders (Table 3.2). In addition, there was no difference in the maximum relaxation to isoprenaline (expressed as percentage of pre-contraction to carbachol) between young and aged bladders on intact bladder tissues or detrusor muscle tissues, but the maximum relaxation to isoprenaline was significantly ($P<0.05$) greater in control urothelial/LP tissues from aged bladders compared to young bladders (Table 3.2).

Effect of doxorubicin pretreatment (Young & Aged)

All tissue strips from doxorubicin pretreated young and aged bladders relaxed to isoprenaline (Figure 3.2). Although, it seems doxorubicin pretreatment greatly reduced relaxation responses of detrusor muscle from aged animals at low concentrations of isoprenaline (but not high), statistical analysis of individual points as shown in Figure 3.2d demonstrated that they are not significantly different. Also, potency of isoprenaline in detrusor muscle from aged bladders was not affected by doxorubicin pretreatment (Table 3.2). In young bladders, doxorubicin pretreatment did not affect either the potency of isoprenaline or the maximum relaxation to isoprenaline in any of tissue (Figure 3.2; Table 3.2).

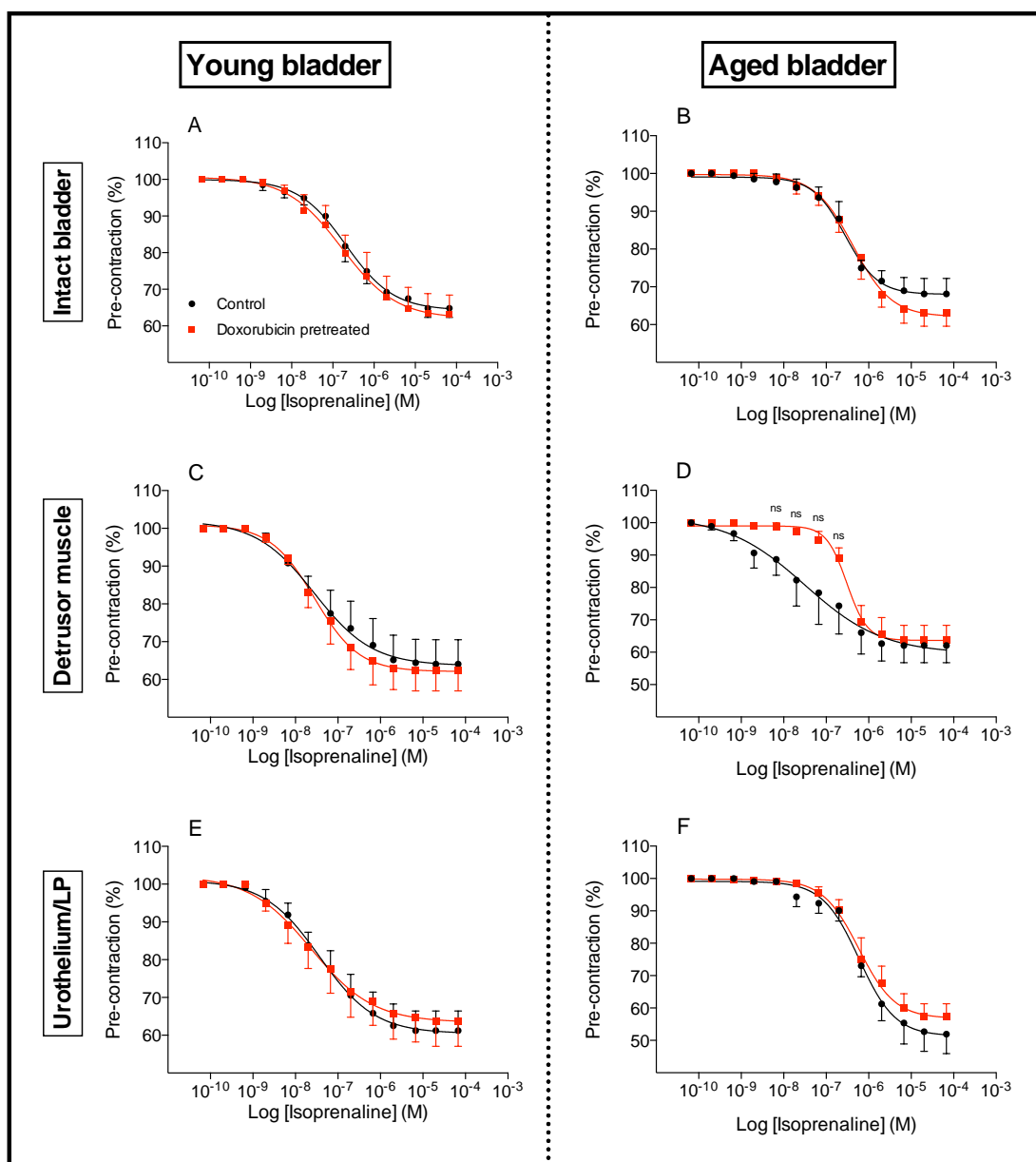


Figure 3.2: Cumulative concentration-response curves to isoprenaline in tissues from control and doxorubicin pretreated bladders from young and aged animals. Responses expressed as a percentage of the pre-contraction to carbachol (27.4 μ M). Responses of intact bladder [A & B], detrusor muscle [C & D], and urothelium/LP [E & F]. ns $P > 0.05$ compared to corresponding responses of control tissue.

Table 3.2: Potency (pEC₅₀) values and maximum relaxation (%) to isoprenaline of intact bladder strips, detrusor muscle strips and strips of urothelium/LP. Data are expressed as means ± SEM.

Young bladders						
Tissue	Control (untreated) bladders			Doxorubicin-pretreated bladders		
	Potency	Maximum	N	Potency	Maximum	N
Intact bladder	6.69±0.12	64.26±1.78	5	6.79±0.24	62.03±3.64	5
Detrusor muscle	7.56±0.29	63.66±3.20	5	7.55±0.18	62.10±2.25	5
Urothelium/LP	7.43±0.21	60.47±2.67	5	7.62±0.33	63.41±3.49	5
Aged bladders						
Intact bladder	6.55±0.11	68.01±1.65	5	6.37±0.11	62.09±2.07	5
Detrusor muscle	7.50±0.45	59.57±5.54	5	6.51±0.09 ^c	63.67±1.66	5
Urothelium/LP	6.23±0.12 ^a	51.35±2.84 ^b	5	6.23±0.11	56.80±2.41	5

N number of bladders examined

^a P<0.01 potency compared to control urothelium/LP from young bladders

^b P<0.05 maximum relaxation compared to control urothelium/LP from young bladders

^c P=0.0631 potency compared to control detrusor muscle

Detrusor responses to electrical field stimulation

Influence of age on responses of untreated (control) tissues

Electrical field stimulation (EFS) induced frequency-dependent contractions of detrusor muscle strips from control incubated young and aged bladders that in preliminary experiments were abolished by the neurotoxin tetrodotoxin (1 μ M), thus confirming their neurogenic origin (data not presented).

At the lowest stimulation frequency of 1Hz, the responses of control tissues from young bladders small (0.003 ± 0.003 mN/mg) (Figure 3.3a) but responses of tissues from aged bladders were greater (0.015 ± 0.005 mN/mg) (Figure 3.3b). With increasing stimulation frequency, responses increased, but at every frequency tested (1, 5, 10 and 20Hz), responses were significantly ($P<0.01$) greater in tissues from aged bladders compared to young bladders (Figure 3.3a and b).

The presence of N(ω)-Nitro-L-Arginine (L-NNA; 100 μ M) failed to potentiate the response in control tissues from young and aged bladders. However, atropine (1 μ M) decreased the detrusor contraction at every frequency and this was similar in both young and aged control tissues (Figure 3.3c and d; Table 3.3). The detrusor contractions were not further reduced by the presence of α,β -mATP (10 μ M) (Figure 3.3c and d).

Effect of doxorubicin pretreatment (Young & Aged)

In detrusor muscle tissues from young and aged bladders that had received doxorubicin pretreatment, a frequency-dependant contraction was observed in response to EFS. Following doxorubicin pretreatment, while the responses to EFS of detrusor strips from young bladders were significantly enhanced at all frequency (Figure 3.3a), responses of tissues from aged bladders were significantly depressed at the higher frequencies of 10 and 20Hz (Figure 3.3b).

The presence of L-NNA (100 μ M) did not potentiate the detrusor contractions to EFS in either young or aged bladders that had received doxorubicin pretreatment, but the responses were depressed by atropine in both age groups (Figure 3.3e and f). The inhibition of responses to EFS by the muscarinic antagonist in doxorubicin pretreated tissues from young bladders and aged bladders were $68.2\pm 4.0\%$ (average of all frequencies) and $52.6\pm 7.8\%$ (average of all frequencies), respectively and these inhibition of responses were not different to those observed in control tissues from

young and aged bladders (Figure 3.3e and f; Table 3.3). The responses were not depressed further by α,β -mATP (Figure 3.3e and f).

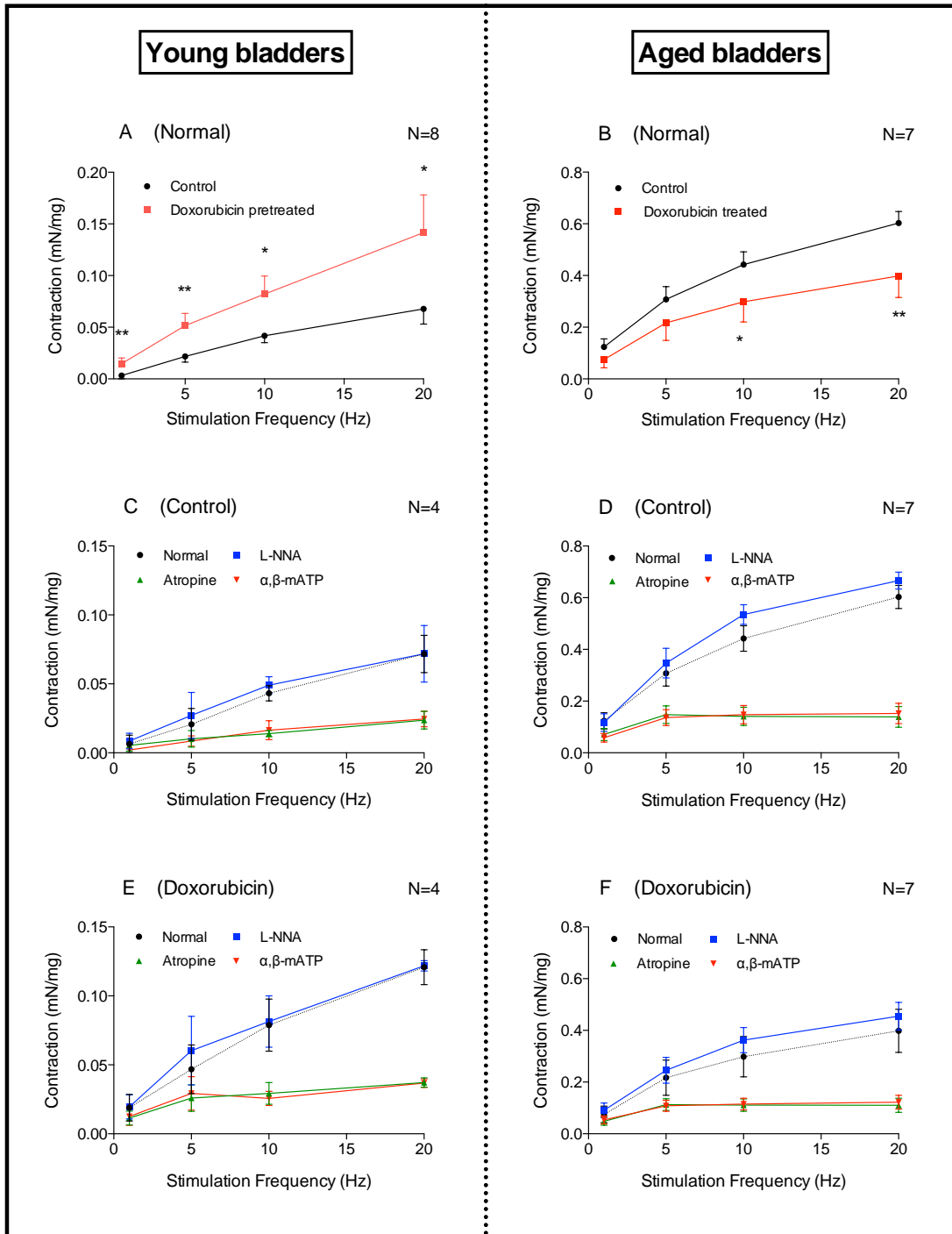


Figure 3.3: Detrusor muscle response to electrical field stimulation (EFS) in tissues from control and doxorubicin pretreated young [A] and aged [B] bladders. Effect of L-NNA, atropine and α,β -mATP on control [C] and pretreated [E] tissues from young animals and control [D] and pretreated [F] tissues from aged animals. Data are expressed as means \pm SEM. * P <0.05 and ** P <0.01 compared to control incubated tissues.

Table 3.3: Reduction (%) in detrusor response to EFS in the presence of atropine (1 μ M). Data are expressed as means \pm SEM.

Young bladders				
Frequency (Hz)	Control (untreated) bladders		Doxorubicin-pretreated bladders	
	Reduction (%)	N	Reduction (%)	N
1	7.69 \pm 7.69	4	41.71 \pm 18.99 ^a	4
5	55.13 \pm 6.17	4	40.66 \pm 6.13	4
10	67.62 \pm 3.71	4	62.85 \pm 2.34	4
20	68.07 \pm 4.32	4	68.20 \pm 4.01	4
<i>Average</i>	<i>49.63\pm14.30</i>		<i>68.20\pm4.01</i>	
Aged bladders				
1	46.76 \pm 9.11	7	42.19 \pm 11.81	7
5	52.11 \pm 18.04	7	37.37 \pm 21.27	7
10	69.40 \pm 17.27	7	59.72 \pm 17.92	7
20	77.28 \pm 17.42	7	71.11 \pm 21.1	7
<i>Average</i>	<i>61.39\pm7.17</i>		<i>52.60\pm7.82</i>	

N number of bladders examined

^a P=0.1479 reduction (%) at 1Hz compared to control bladders.

Urothelial mediators

Influence of age on responses of untreated (control) tissues

Urothelial/LP strips from young and aged bladders that received control incubation released all three mediators (ATP, ACh and PGE₂) (Figure 3.4 and 3.5).

The release of urothelial/LP mediators was measured at 2, 3 and 7 minute time points in tissues from young bladders but in tissues from aged bladders mediator release was measured at four different time points including 1, 2, 3 and 7 minutes. The difference in time points was due to the fact that only three time points were selected for measuring mediator release from young bladders during my Honours study whilst for my PhD study, the 1 minute time point was also included to provide additional information. Thus, when comparing the release between young and aged bladders, only 2, 3 and 7 minute time points will be considered.

Stretching the control tissues from young bladders increased ATP release at 2, 3 and 7 minute time points, but the increases were not statistically different (Figure 3.4a and b). Stretch of the tissues from aged bladders failed to increase any of the mediators at any time point (Figure 3.5a and b).

In control tissues, basal release of ATP from aged bladders was significantly enhanced compared to young bladders at 2, 3 and 7 minute time points (Figure 3.6a). Stretch-induced ATP release from aged bladders was also increased significantly compared to aged bladders but only at 3 and 7 minutes (Figure 3.6b). Both basal and stretch-induced release of ACh and PGE₂ from aged bladders were significantly enhanced compared to young bladders at 2, 3 and 7 minutes (Figure 3.6c, d, e and f).

The average values of basal ATP, ACh and PGE₂ release at 2, 3 and 7 minute time points in control tissues from aged bladders were increased by 9-fold, 9-fold and 4-fold compared to the values from young bladders (Table 3.4). The average values of stretch-induced ATP, ACh and PGE₂ release at the same time points from aged bladders were increased by 2-fold, 8-fold and 4-fold compared to the values from young bladders (Table 3.4).

Effect of doxorubicin pretreatment (Young & Aged)

Doxorubicin pretreated tissues from both young and aged bladders also released all three mediators and stretching the tissues only produced increase in ATP release at 2,

3 and 7 minutes in young bladders and at 7 minutes in aged bladders (Figure 3.4 and 3.5).

In doxorubicin-pretreated tissues from young bladders, basal release of ACh and PGE₂ was similar to control tissues (Figure 3.4c, d, e and f). However, basal release of ATP in pretreated tissues was significantly increased at 2, 3 and 7 minutes compared to control tissues (Figure 3.4a). The release of stretch-induced ATP was also significantly increased at 3 and 7 minutes (Figure 3.4b).

In tissues that received doxorubicin pretreatment from aged bladders, basal release of all three mediators was similar to control tissues (Figure 3.5), but stretch-induced ATP release was significantly increased compared to control tissues at 7 minutes (Figure 3.5b).

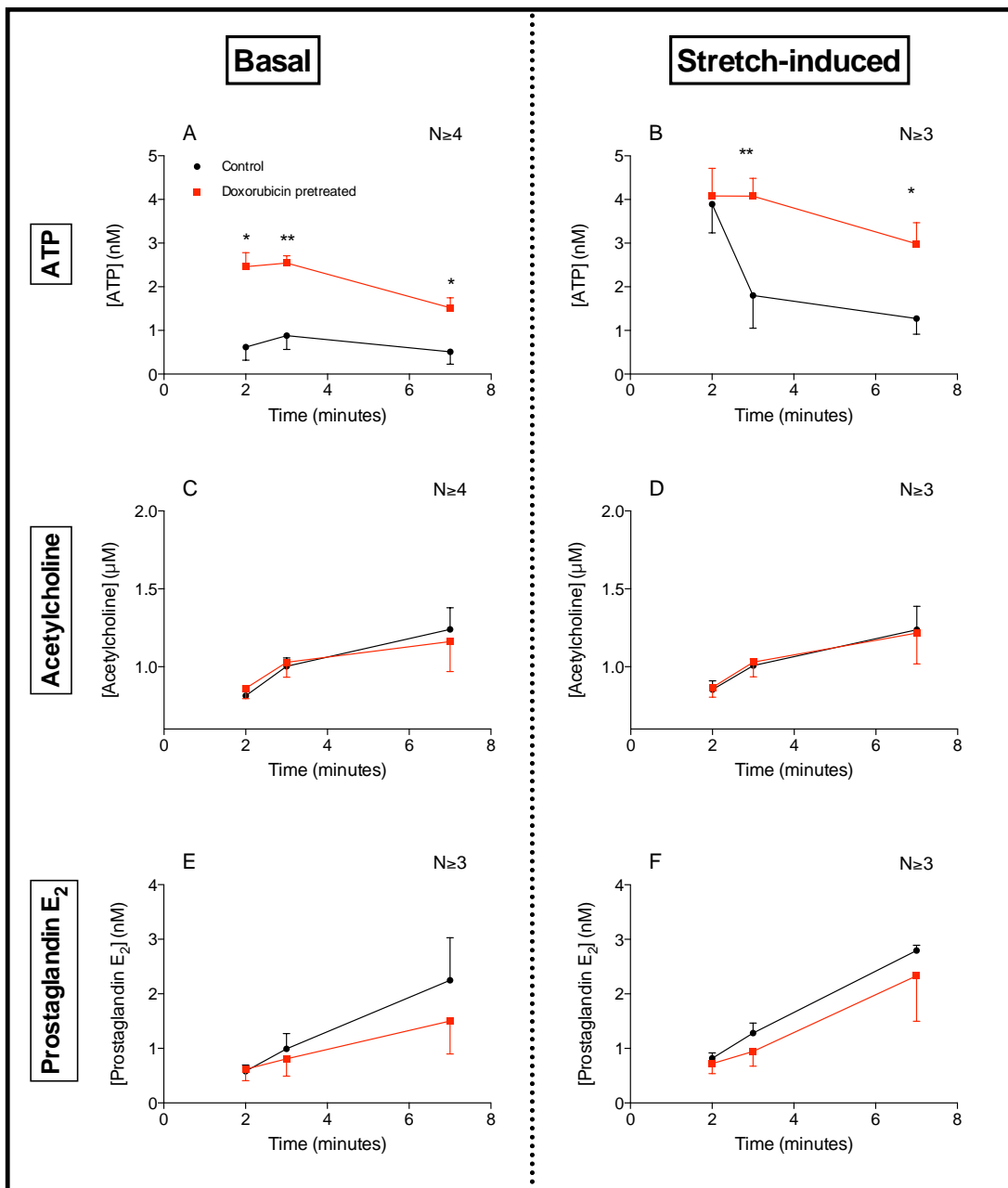


Figure 3.4: Mediator release from the urothelium/LP of young bladders. Effects of doxorubicin pretreatment on basal ATP [A], acetylcholine [C] and prostaglandin E₂ [E] concentrations after 7 minute incubation, and ATP [B], acetylcholine [D] and prostaglandin E₂ [F] concentrations after 7 minute incubation that included stretching over the initial 2 minutes. Data are expressed as means \pm SEM. *P<0.05 compared to control incubated tissues.

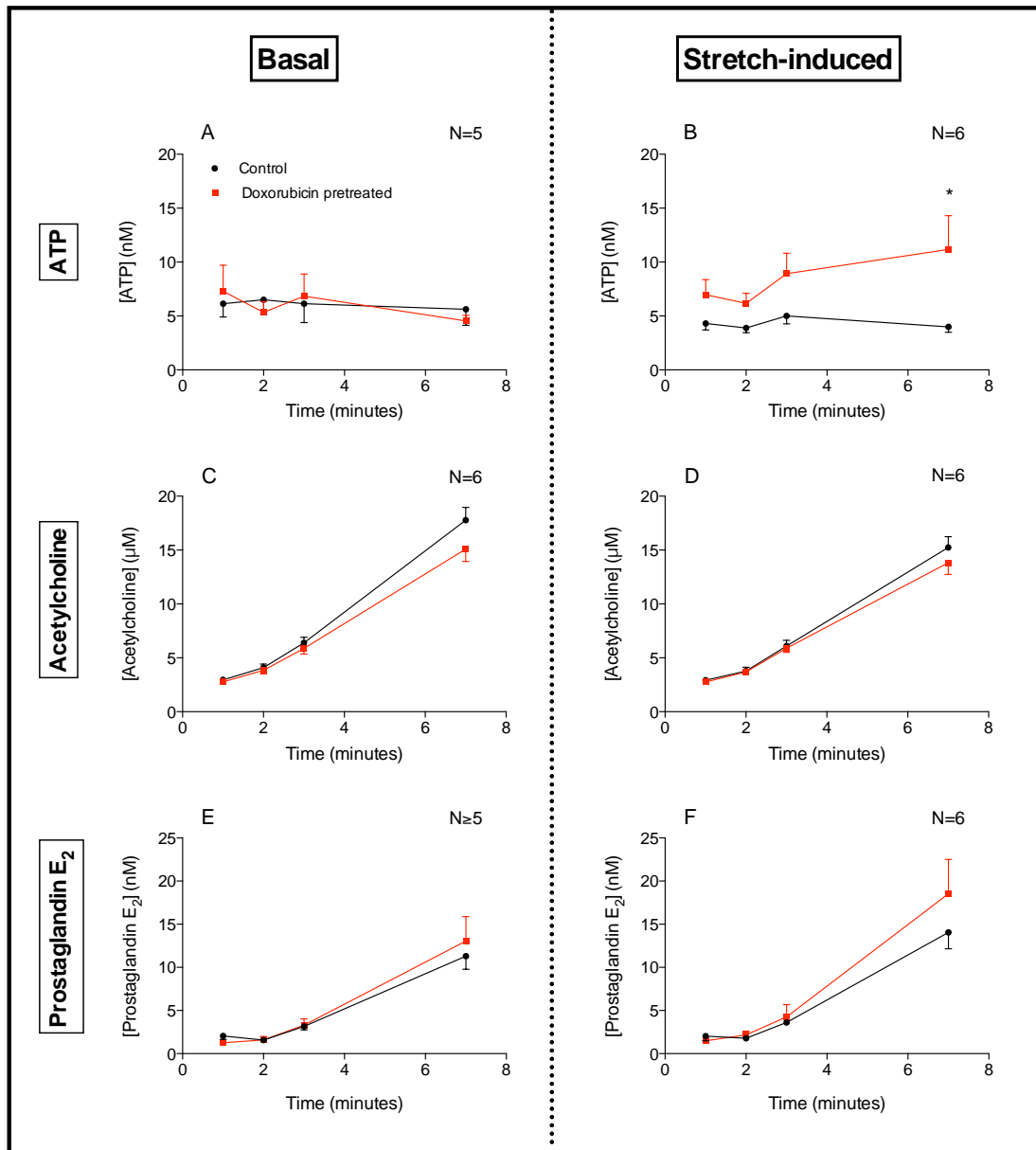


Figure 3.5: Mediator release from the urothelium/LP of aged bladders. Effect of doxorubicin pretreatment on basal ATP [A], acetylcholine [C] and prostaglandin E₂ [E] concentrations after 7 minute incubation, and ATP [B], acetylcholine [D] and prostaglandin E₂ [F] concentrations after 7 minute incubation that included stretching over the initial 2 minutes. Data are expressed as means \pm SEM. *P<0.05 compared to control incubated tissues.

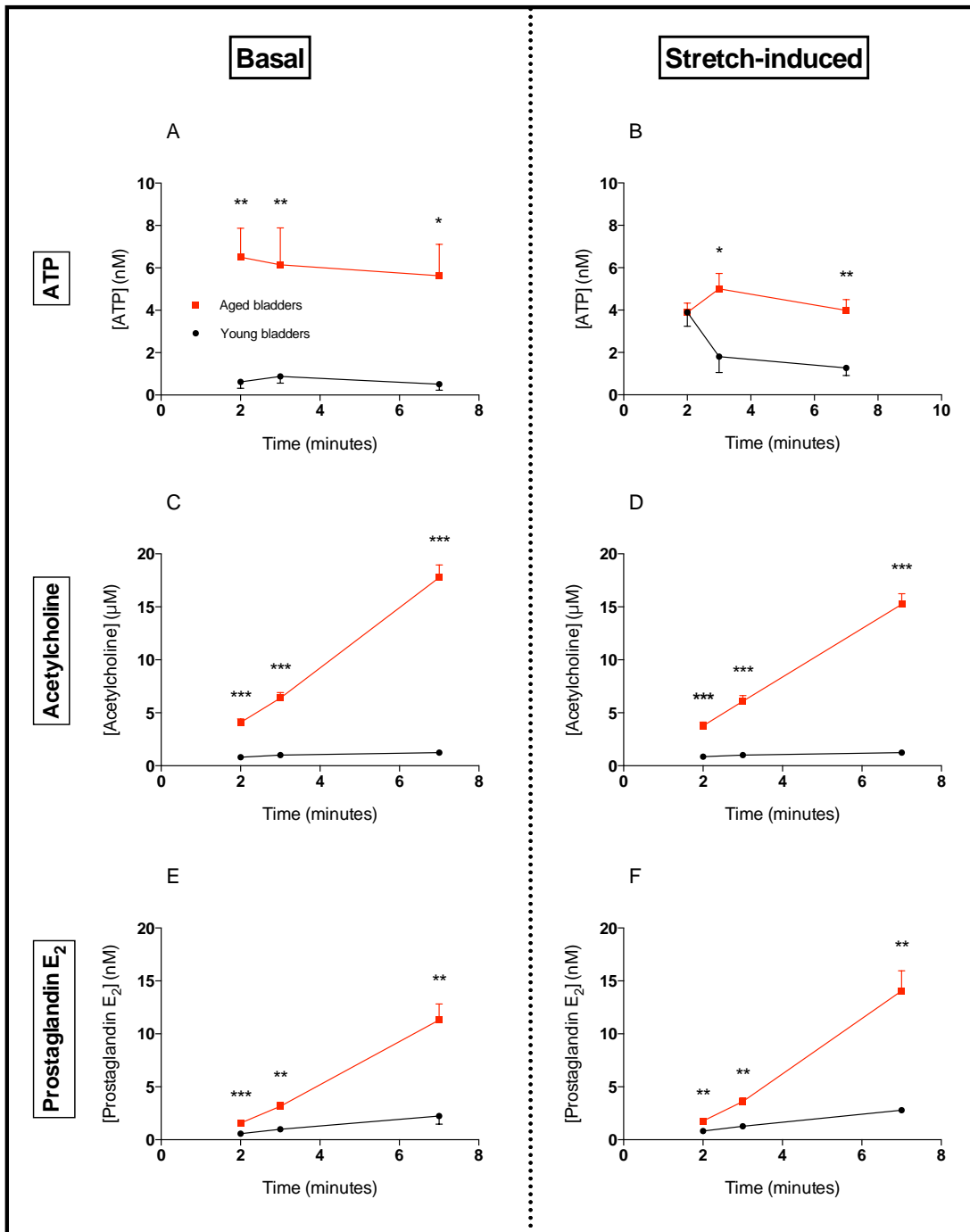


Figure 3.6: Comparison of urothelial/LP mediator release in control tissues from young and aged bladders. Basal and stretch-induced release of ATP [A & B], acetylcholine [C & D], and prostaglandin E₂ [E & F] concentrations after 7 minute incubation. Data are expressed as means \pm SEM. *P<0.05 compared to young bladders.

Table 3.4: Mean (\pm SEM) urothelial/LP mediator concentrations at 2, 3 and 7 minute time points from control incubated tissues.

Young bladders		
Mediator	Basal release	Stretch-induced release
	Concentration	Concentration
<i>ATP</i> (nM)	0.67 \pm 0.11	2.32 \pm 0.8
<i>ACh</i> (μ M)	1.02 \pm 0.12	1.03 \pm 0.11
<i>PGE</i> ₂ (nM)	1.28 \pm 0.5	1.63 \pm 0.59
Aged bladders		
<i>ATP</i> (nM)	6.09 \pm 0.25	4.29 \pm 0.36
<i>ACh</i> (μ M)	9.42 \pm 4.23	8.37 \pm 3.49
<i>PGE</i> ₂ (nM)	5.35 \pm 3.01	6.48 \pm 3.83

Inhibition of detrusor contraction by the urothelium

Influence of age on responses of untreated (control) tissues

The presence of an intact urothelium/LP on strips of detrusor muscle inhibited contractions to carbachol in control tissues from both young and aged bladders (Figure 3.7a and b). While the potency of carbachol was similar in intact bladder and detrusor muscle tissues, maximum responses were significantly depressed ($P<0.01$; $21.9\pm 11.7\%$) in aged bladders. In contrast, whilst maximum responses of young bladders look significantly depressed ($32.9\pm 15.8\%$) in tissues with intact urothelium/LP compared to tissues without the urothelium/LP, statistical analysis revealed that it was not the case. The degree of inhibitions was similar in young and aged bladders.

Effect of doxorubicin pretreatment (Young & Aged)

The presence of urothelium/LP on detrusor muscle that had received doxorubicin pretreatment from young and aged bladders also inhibited contractions to carbachol (Figure 3.7c and d). The potency of carbachol was again similar in intact bladder and detrusor muscle tissues, but the maximum responses were significantly reduced ($P<0.01$ for young bladders; $P<0.001$ for aged bladders) in tissues with an intact urothelium/LP. The inhibition was similar in tissues from both young ($30.1\pm 21.9\%$) and aged ($36.1\pm 12.8\%$) bladders and the amount of inhibition was not affected by doxorubicin pretreatment in both young ($P=0.9178$) and aged ($P=0.432$) bladders.

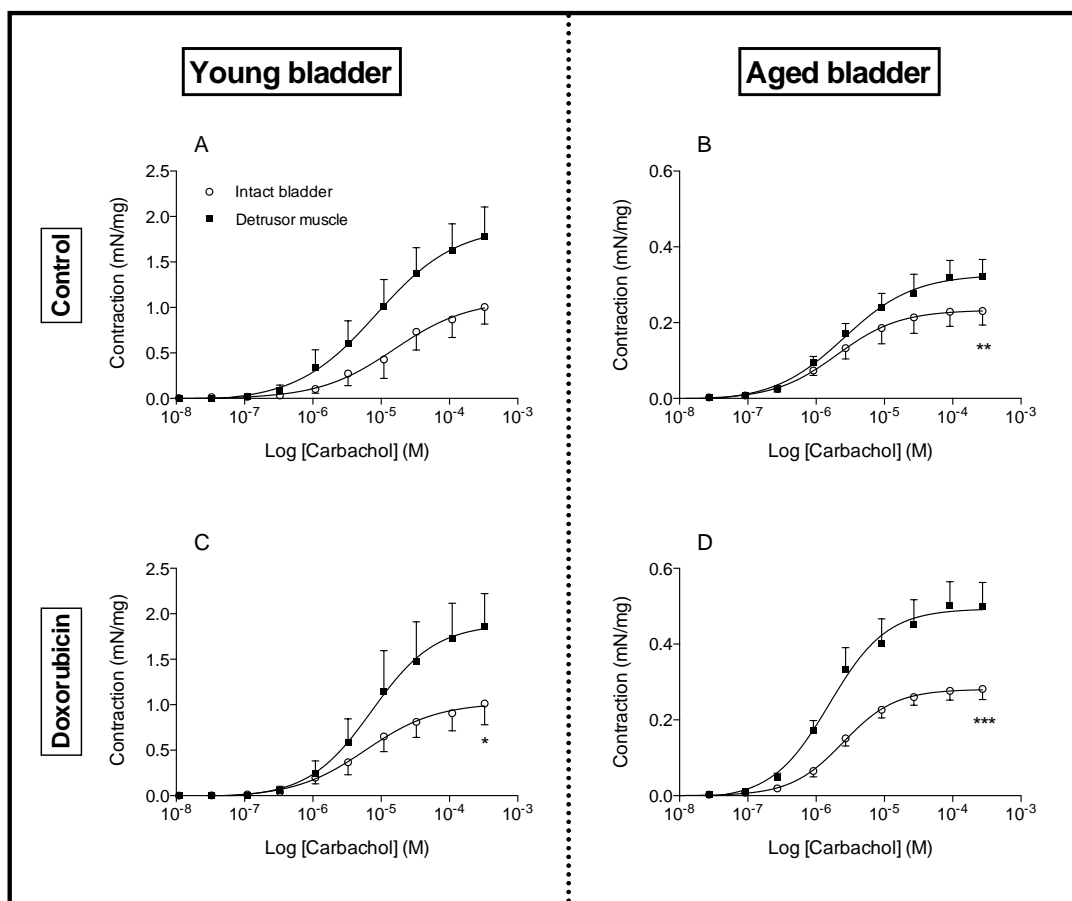


Figure 3.7: Inhibitory effects of the urothelium/LP on detrusor responses to carbachol in intact bladder and detrusor muscle tissues from control [A & B] and doxorubicin pretreated [C & D] bladders. Data are expressed as means \pm SEM. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared to maximum response of detrusor muscle.

Bladder histology

Influence of age on responses of untreated (control) tissues

Typical histological features were clearly identifiable in sections of control incubated young and aged bladders, with the urothelium and lamina propria thrown into folds and overlying a deeper detrusor muscle layer (Figure 3.8 and 3.9). Urothelial thickness of young bladders ($35.04 \pm 2.21 \mu\text{m}$, N=4) was significantly thicker ($P < 0.05$) than the aged bladder ($27.81 \pm 1.94 \mu\text{m}$, N=4).

Effect of doxorubicin pretreatment (Young & Aged)

In sections of doxorubicin pretreated young and aged bladders, typical histological features were also clearly observed (Figure 3.8 and 3.9). Doxorubicin treatment did not affect urothelial integrity with the urothelial thickness of pretreated young ($33.18 \pm 3.03 \mu\text{m}$, N=4) and aged ($27.48 \pm 2.62 \mu\text{m}$, N=4) bladders being similar to those of control incubated young and aged bladders.

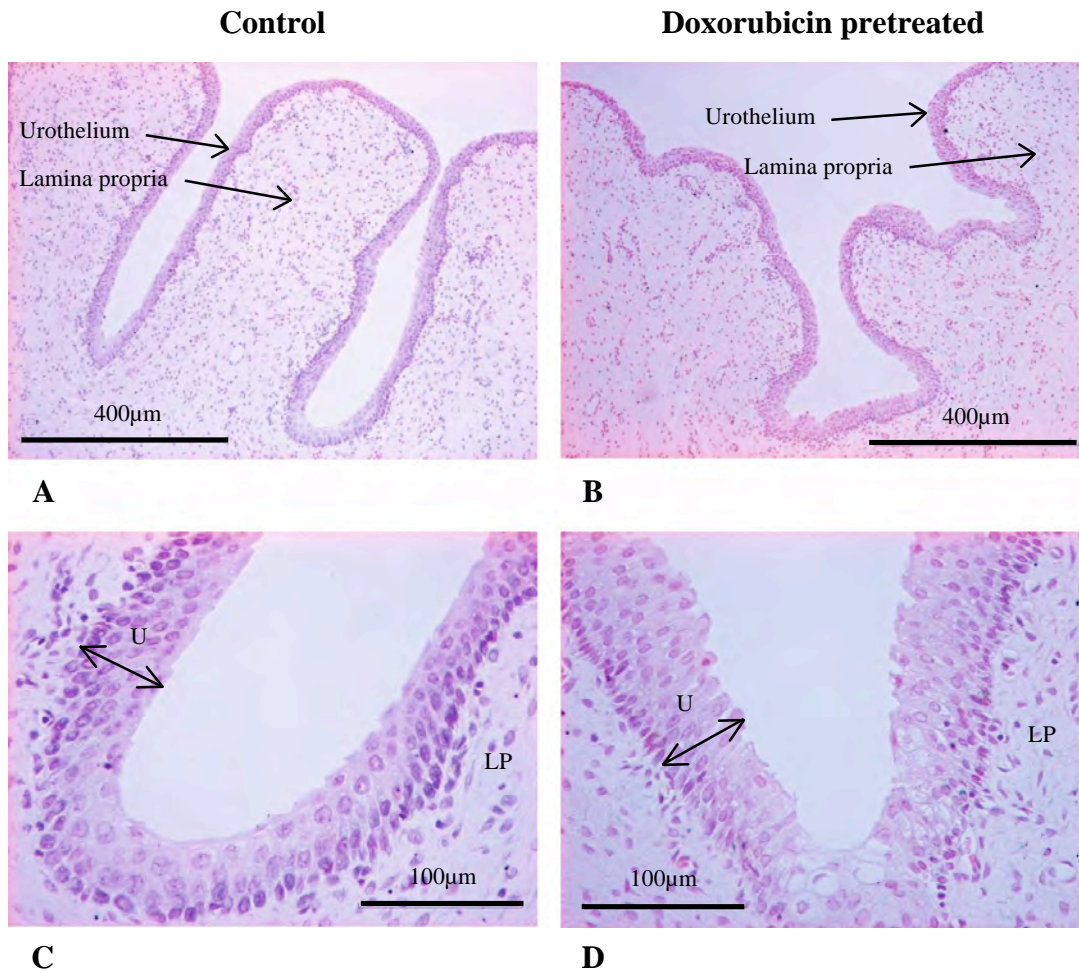


Figure 3.8: Haematological examination of young bladder section. Haematoxylin and eosin (H and E) staining of control [A & C], and doxorubicin pretreated [B & D] bladders. H and E staining at x10 [A & B] and x40 [C & D] showing the urothelium (*U*) and lamina propria (*LP*).

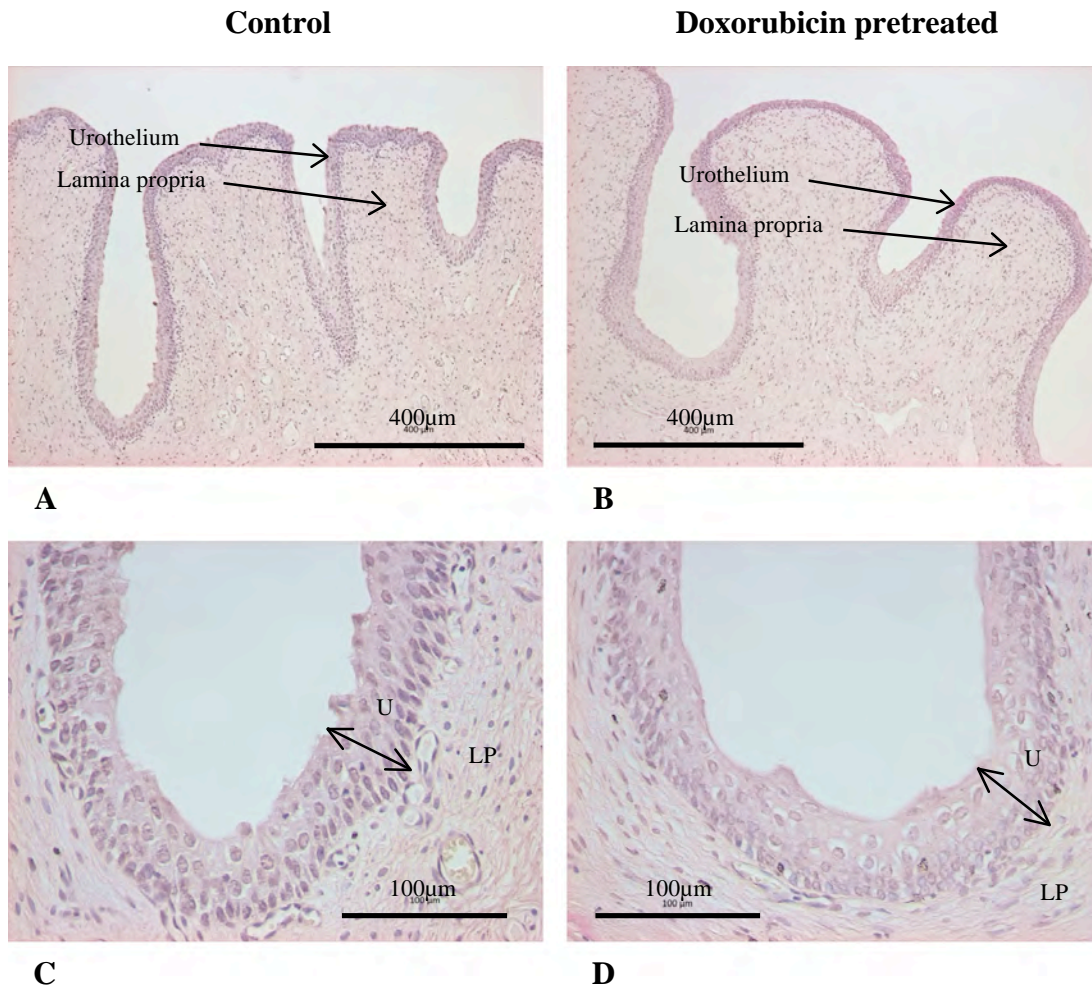


Figure 3.9: Haematological examination of aged bladder section. Haematoxylin and eosin (H and E) staining of control [A & C], and doxorubicin pretreated [B & D] bladders. H and E staining at x10 [A & B] and x40 [C & D] showing the urothelium (U) and lamina propria (LP).

Discussion

Mediator release from the urothelium/LP

ATP, ACh and PGE₂

In this study, a basal release of ATP was observed in control and doxorubicin pretreated urothelium/LP tissues from both young and aged bladders. In young bladders, basal ATP release was enhanced in tissues that received doxorubicin pretreatment compared to control tissues. Stretching the tissues increased ATP release over basal levels in both control and pretreated tissues and this stretch-induced ATP release was increased following doxorubicin pretreatment. In aged bladders, while doxorubicin pretreatment did not affect basal ATP release, stretch-induced ATP release was significantly enhanced in pretreated tissues.

It is well established that in normal bladder, the urothelial ATP initiates a mechanism by which low threshold sensory nerves are stimulated via P2X₃ receptors on the urothelium and lamina propria and thereby activates the micturition reflex (Burnstock, 2011). Stretch-induced ATP release is increased in the bladders of patients with idiopathic or neurogenic OAB (Kumar et al., 2010). Various *in vitro* studies using animal models of OAB and IC have also suggested that increased urothelial ATP release from damaged or sensitised cells in response to injury or inflammation might be the contributing factor for triggering increased bladder activity and painful sensations via excitation of purinergic (P2X) receptors on sensory fibers (Khera et al., 2004; Munoz et al., 2011; Smith et al., 2005; Sun et al., 2001). In addition, reduced ecto-ATPase activity was observed in OAB (Nishiguchi et al., 2005), which suggests that intravesical doxorubicin treatment could possibly increase ATP levels by acting on ecto-ATPase (Moore et al., 1992). Thus, elevated ATP release from both young and aged bladders in this study may provide an explanation for the increased urgency and frequency observed in patients treated with intravesical doxorubicin. In addition, ATP can also activate high threshold, nociceptive (pain) nerve fibres and ATP release is also greatly increased in patients with painful bladder syndrome (Kumar et al., 2007). Thus, the dysuria observed after intravesical treatment with doxorubicin may be related to the enhancement of urothelial ATP release.

There was also basal release of ACh and PGE₂ from the urothelium/LP, but unlike ATP, stretch did not increase release. The role of these urothelial mediators is not completely elucidated yet, but ACh appears to influence sensory nerve activity (Daly et al., 2010; Yu and de Groat, 2010) and urothelial contractile activity (Akino et al., 2008; Moro et al., 2012; Moro et al., 2011), while PGE₂ influences bladder contractions (de Jongh et al., 2007) and sensitizes some capsaicin-sensitive afferent neurons (Maggi et al., 1988a). A stretch-independent release of this prostaglandin from the urothelium has also been reported (Nile et al., 2010). Doxorubicin pretreatment altered neither the basal nor the stretch-induced release of ACh and PGE₂ in either young or aged bladders and it is therefore unlikely that these mediators are involved in the adverse effects induced by intravesical administration of this cytotoxic drug.

The finding that doxorubicin affected the mediators differently is not surprising since the release mechanisms for these mediators is very different. Prostaglandins including PGE₂ are synthesised and released fresh by cyclooxygenase (COX)-1 and COX-2 in the bladder during detrusor contraction (Bultitude et al., 1976; Khalaf et al., 1980; Klarskov, 1987) and under basal physiological conditions (Brown et al., 1980; Poggesi et al., 1980). The expression of COX-1 by the basal layer of the urothelium indicates that prostaglandins are synthesised and released from these sites (de Jongh et al., 2009; Rahnama'i et al., 2010). Many potential mechanisms for urothelial-derived ATP release have been proposed including vesicular release (Birder et al., 2003; McLatchie and Fry, 2014; Sui et al., 2014; Wang et al., 2005), connexin or pannexin hemichannels (McLatchie and Fry, 2014; Timoteo et al., 2014; Wang et al., 2005), transient receptor potential channels (TRPV) 1 or 4 (Mochizuki et al., 2009; Wang et al., 2005), ATP-binding cassette (ABC) transporters (Wang et al., 2005) and mechanosensitive channels (Birder et al., 2003; Dunning-Davies et al., 2013). A recent study by McLatchie et al. (2014) has suggested the potential involvement of the cystic fibrosis transmembrane conductance regulator (CFTR) and intracellular calcium in urothelial-derived ACh release and it appears to be independent of extracellular calcium, connexins and pannexins, vesicular acetylcholine transporter (VACHT) or the organic cation transporter (OCTs) 1 and 3 (Lips et al., 2007; McLatchie et al., 2014).

The amount of all three mediators, both basal and stretch-induced release was greater in tissues from aged bladders compared to tissues from young bladders. The observation of age-related increase in mediators from the urothelium/LP in this study was consistent with some previous studies, although other studies are contradictory. A study by Yoshida et al. (2006) using human bladder strips with or without urothelium has shown that the tetrodotoxin-insensitive non-nerve-evoked ACh release was significantly greater in strips with urothelium than without urothelium and this non-nerve-evoked ACh release from strips with urothelium increased with age. Also, an age-related increase in the stretch-induced release of non-nerve-evoked ACh was observed. Similarly for ATP, a recent study with mice, Daly et al. (2014) demonstrated significantly increased ATP levels in the lumen of aged bladder (24 months) preparations compared to young bladders (3-4 months) when the whole intact bladder was distended and also significantly reduced ACh levels were reported in aged bladders in comparison to young bladders. Furthermore, the PGE₂ levels in urine samples collected from young boys (aged less than 15 years old) with OAB was negatively correlated with age (N=38, $r = -0.379$, $P < 0.05$) (Aoki et al., 2009). The results of these studies suggest that ageing may alter the release of mediators from the bladder including the urothelium.

Urothelium-Derived Inhibitory Factor

The urothelium/LP from healthy bladder also releases unidentified substances during muscarinic receptor stimulation that inhibits contraction of the underlying detrusor smooth muscle in the pig and human bladder. As a result of these unknown substances, bladder overactivity and consequent involuntary urine loss may be prevented (Chaiyaprasithi et al., 2003; Hawthorn et al., 2000; Templeman et al., 2002). This urothelium-derived inhibitory factor (UDIF) is not NO or a prostaglandin and it remains unidentified.

The influence of this factor was noticeable in the present study, where the presence of an intact urothelium/LP significantly depressed maximum contractions of the detrusor muscle to carbachol in both young and aged bladders.

After doxorubicin pretreatment the responses of intact bladder and detrusor muscle tissues to carbachol and the degree of inhibition by the urothelium/LP was similar to the tissues from untreated bladders. Thus, doxorubicin did not appear to alter either

the release or the actions of UDIF and its effects on mediator release from the urothelium/LP were limited to an action on ATP release.

Tissue responses

Muscarinic-mediated contractile response

In all tissues examined (in both young and aged bladders), the muscarinic agonist carbachol induced contraction. These were greater in urothelial/LP strips than those produced by strips of detrusor muscle and intact bladder when responses were expressed relative to weight (mN/mg tissue). This finding is similar to what was observed in a previous study by Sadananda et al. (2008) where strips of urothelium containing lamina propria but no detrusor muscle from pig bladder produced significantly greater contractile response to carbachol compared to the response produced by intact bladder strips. However, the magnitude of responses in urothelial/LP strips was not significantly different from responses in detrusor muscle strips.

Following pretreatment with doxorubicin, contractile responses of urothelial/LP strips from both young and aged bladders were enhanced. The potency of carbachol was unaltered, but the responsiveness of the tissues was increased and the maximum responses were significantly greater following doxorubicin pretreatment. However, the relevance of this finding to the development of OAB is currently unclear. In a report by Kanai et al. (2007), it was demonstrated that spontaneous membrane potential transients induced by stretch can originate in the urothelium/LP. Also, in feline IC cat bladders, there was increased calcium transient in the urothelium/LP which spread to the detrusor muscle in tissues from animals with IC (Ikeda et al., 2009). These findings suggest that it is possible that contractions generated spontaneously in the urothelium/LP can cause spontaneous contractions of detrusor smooth muscle. Thus, it is possible that the increase in contractile activity of this layer (urothelium/LP) after doxorubicin pretreatment may contribute to the bladder overactivity observed in patients following treatment. This idea is supported by the finding that gap junction density is increased in patients with urge symptoms. A previous study by Neuhaus et al. (2005) compared the expression of connexin (Cx)40, Cx43 and Cx45 in bladder biopsies from a control group and an incontinence group.

Control tissues were collected from bladder cancer patients without previous history of LUTS who underwent cystectomies or TUR. Control tissue samples isolated from the bladder were free from bladder carcinoma. The incontinence group consisted of patients with urge urinary incontinence, mixed urinary incontinence and painful bladder syndrome. The study demonstrated significantly higher Cx43 expression in the detrusor muscle, and a tendency to higher Cx45 expression in the LP layer associated with urge symptoms, while Cx40 expression was unaffected.

The effects of doxorubicin on detrusor muscle contraction were also examined. It was reported that less than 1% of doxorubicin penetrates this far into the bladder wall (Wientjes et al., 1996) and not surprisingly the responses of the muscle itself to muscarinic stimulation from young bladders were unaltered by doxorubicin pretreatment. However, the response was enhanced in aged bladders following doxorubicin pretreatment. The potency of carbachol was unaltered, but the responsiveness of the detrusor muscle tissue was increased and the maximum responses were significantly greater following doxorubicin pretreatment. The enhanced detrusor contractile responses to carbachol observed in aged bladders following doxorubicin treatment may be due to upregulation of the calcium sensitisation pathway such as Rho kinase (ROK) and protein kinase C (PKC). The mechanisms responsible for initiation and maintenance of smooth muscle contractions, induced by stimulation of G protein-coupled receptors (GPCRs) are thought to be an increase in cytosolic calcium produced by calcium entry through voltage sensitive calcium channels, or calcium release from the sarcoplasmic reticulum (SR) (Durlu-Kandilci and Brading, 2006). Muscarinic receptor stimulation in detrusor smooth muscle has been reported to increase intracellular calcium through release of intracellular calcium from IP₃ or ryanodine sensitive stores as well as from influx of extracellular calcium (Ma et al., 2002; Visser and van Mastrigt, 2000). Activation of GPCRs by agonists increases force in smooth muscle via calcium-dependent myosin light chain phosphorylation. The level of myosin light chain phosphorylation and the activation of smooth muscle are determined by the ratio of activities of calcium/calmodulin dependent myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP). However, there are mechanisms that can cause muscle contractions without any necessary change in intracellular calcium. When intracellular calcium and MLCK activity are constant, agonist activating GPCRs may cause a leftward shift of the calcium/force response

curve which is known as calcium sensitisation (Durlu-Kandilci and Brading, 2006). There are two major pathways known to be responsible for calcium sensitisation which includes ROK and PKC (Horowitz et al., 1996; Somlyo and Somlyo, 2000) and they are thought to be involved in inhibition of myosin phosphatase activity at constant calcium and therefore enhance myosin light chain phosphorylation and induce an increase in contractile force (Somlyo and Somlyo, 2003). A study by Rajasekaran et al. (2005) has demonstrated that bladder hyperactivity in spontaneously hypertensive rats (SHR) is attenuated by inhibition of RhoA/Rho-kinase with Y-27632 and also reported increased bladder expression of RhoA in SHR, suggesting that bladder contractions and tone in SHR are modulated in part by RhoA/Rho-kinase calcium sensitizing pathway. Thus, it is possible that the increase in contractile activity of both urothelium/LP and the detrusor muscle after doxorubicin pretreatment may contribute to the overactivity observed in patients treated intravesically with doxorubicin.

There is no study in the literature demonstrating the difference in the severity of adverse effects induced by intravesical chemotherapy including doxorubicin in aged versus young. While the effect of ageing on drug permeability into the bladder layers is unknown, at least in this study, doxorubicin pretreatment seems to be exerting greater toxicity in aged bladders since not only the contractile response of the urothelium/LP to muscarinic stimulation was enhanced but also the response of the deeper underlying detrusor muscle was increased in the aged bladders following doxorubicin pretreatment. It seems that doxorubicin has greater cytotoxic effects on aged bladders, possibly due to the declining barrier function of the bladder with age as previously discussed in the introduction of this chapter. There are several studies reporting greater severity of side-effects in aged patients in other physiological systems resulting from systemic use of chemotherapeutic drugs. A regression analysis study by Begg, Elson & Carbone (1989), which calculated the odds ratio (OR) of cytotoxic drug doxorubicin for grade 3-4 hematological toxicity in elderly patients (8787 patients aged <60 years, 5584 aged 60-69 years and 2209 aged ≥ 70 years), demonstrated a higher risk of severe hematological toxicity for patients at the age of ≥ 70 years (OR=1.4). A recent study by Goto et al. (2012) also reported an increased incidence of side-effects in elderly (aged 65 years or older) cancer patients treated with chemotherapy compared to younger patients (younger than 65 years). Myelosuppression of grade 3 or more severe adverse effects in the elderly patients and

younger subjects was 22.5% and 16.3%, respectively. Cardiotoxicity is a major limiting factor with the use of doxorubicin and age is one of the established risk factors for doxorubicin-induced cardiotoxicity (Von Hoff et al., 1979).

In this study, an age-related decrease in contractile response to muscarinic stimulation was observed. The maximum contractions to carbachol in all control tissues were significantly smaller in aged bladders compared to young bladders. In addition, potency of carbachol was lower in intact bladder and urothelium/LP tissues from aged bladders compared to young bladders. These findings may indicate that tissue responsiveness and sensitiveness to muscarinic stimulation diminish with age. A study by Toyoshima et al. (1990) also supports the view of reduced responsiveness of the bladder tissues to muscarinic stimulation with age since maximal response in intravesical pressure during bladder contraction induced by ACh was lower in aged rats (2 years old) compared to younger adult rats (11-23 weeks old). In contrast, there are studies reporting increased contractile response to muscarinic stimulation in aged bladders compared to young bladders. A study by Kolta, Wallace & Gerald (1984) has shown that the maximum contractile responses to ACh were 64% and 15% greater in isolated bladders from 29 months and 17 months rats, respectively, as compared to 7 months rats and the density of [³H] quinuclidinyl benzilate (muscarinic antagonist) bound to membrane preparations were 46% and 7% greater. This study suggests that urinary bladder of aged rats appears to develop increased responsiveness to cholinergic stimuli and increase in the number of muscarinic cholinergic receptors. Another study has also demonstrated an age-dependent increase in the maximum contractions elicited by muscarinic agonists where the maximum contraction values for strips of bladder base from rats 27 months of age were 44%, 58% and 76% greater than those from rats 7 months of age for ACh, bethanechol and oxotremorine, respectively (Ordway et al., 1986). In addition, a recent study by Daly et al. (2014) reported significantly increased ($P<0.001$) contractile responses of detrusor muscle strips from aged mice (24 months old) to bethanechol compared to muscle strips from control mice (3 months old). In contrast, there are studies that found no change in contractile responses of the bladder body (Lluel et al., 2000) and base (Longhurst et al., 1992) to carbachol between young and aged rats. Whilst there are contrasting reports in the literature about the effects of age in rats and mice, this study was first to report an aged-related changes in contractile response to muscarinic stimulation in pig which may be more relevant to human.

Nerve-mediated contractile response

Both young and aged detrusor muscle tissues responded to EFS at all frequencies tested and these neurogenic responses were found to be significantly greater in tissues from the aged bladders when compared to the responses in young bladders. These findings, which suggest an aged-associated increase in contractile response of detrusor muscle to efferent nerve stimulation, are consistent with the previous studies. In a report by Longhurst et al. (1992), EFS produced a greater contractile response in the bladder body of old rats (24 months old) compared to young rats (6 months old). Another study also demonstrated significantly larger contractile responses in circular detrusor fibers evoked by EFS in old (27 months old) rats compared with those of young (6 months old) rats (Pagala et al., 2001). However, a study by Munro & Wendt (1993) using longitudinal smooth muscle strips from bladders of rats reported no significant difference in contractile response to EFS between adult (6 months old) and aged (24 months old) bladders. In addition, Yoshida et al. reported similar finding in their study in which EFS-induced contractions in detrusor muscle from human bladder were not significantly different among the three age groups they examined (G1, <50 years old; G2, 51-70 years old; G3, >70 years old) (Yoshida et al., 2001).

Following doxorubicin pretreatment, contractile responses of detrusor muscle to EFS were enhanced in young bladders. It can be suggested that in young bladders, doxorubicin treatment does not affect the detrusor muscle directly, but does affect the efferent nerves, increasing transmitter release and thus enhancing neurogenic detrusor contractions. Therefore, it can be postulated that the mechanism by which doxorubicin enhances transmitter release may induce detrusor contractions during bladder filling and contribute to bladder overactivity. In contrast, in the aged bladders, doxorubicin treatment depressed neurogenic responses of detrusor muscle in addition to an enhanced contractile responsiveness of detrusor muscle. It appears that doxorubicin causes a greater cytotoxicity in the aged bladders compared young bladders since both the muscle and efferent nerves are affected by the treatment. In addition, it appears that in the aged bladders, doxorubicin treatment may cause overactivity of the bladder via directly acting on detrusor muscle contractility rather than enhancing neurotransmitter release as in young bladders since the depressed neurogenic responses observed in aged bladders after the treatment may indicate toxic damage on efferent nerves. The neurotoxicity observed in this study may be

due to the effects of ageing on the bladder function which can lead to greater susceptibility of aged bladders to doxorubicin toxicity. There is a study by Gottlieb et al. (1987) which supports this view where he claimed that neurotoxicity is age related. In the study, fifteen patients with acute myeloid leukemia were given a total of 17 courses of high-dose cytosine arabinoside (Ara-C) and of five patients over the age of 55 years, four developed severe, irreversible neurotoxicity, but there were no severe episodes in patients aged 55 years or less ($P < 0.01$), suggesting that administration of high dose chemotherapy Ara-C carries a risk of severe irreversible cerebellar toxicity that increases with age.

In both pig and human bladder, the cholinergic component plays a major role in nerve-mediated detrusor muscle contraction (Yoshida et al., 2004; Yoshida et al., 2002). Although, less pronounced compared to cholinergic responses, purinergic neurotransmitters are also involved in detrusor muscle contraction (Burnstock, 1986; Hoyle et al., 1989). Our study also agree that the cholinergic neurotransmission is the major pathway for detrusor muscle contraction, as contractile response of detrusor muscle to EFS in both young and the aged bladders were reduced in the presence of atropine, but did not abolish responses which is consistent with a finding by Sibley (1984) that observed inhibition of contractile response of pig intact bladder tissue to EFS by 22% in the presence of atropine (0.01-1nM). However, when P_2 -purinoceptors were desensitised with α,β -mATP in the present study, neurogenic responses were not affected. A study by Brading & Williams (1990) using rat detrusor muscle strips also found a small but not significant reduction in response to stimulation at 1Hz and 25% reduction in the responses were observed at 20Hz in the presence of atropine. Their study also reported completely abolished responses at 1Hz and 75% reduction at 10 and 20Hz in the presence of α,β -mATP. In addition, combination of both drugs eliminated the nerve-mediated responses. The differences observed may be attributed to species-dependent neurotransmitter responses.

The effects of atropine were not affected by doxorubicin treatment in either young or aged, suggesting that doxorubicin affects cholinergic and purinergic neurotransmission equally when neurotransmission is enhanced in young pigs following treatment with doxorubicin. In addition, no differences were observed in cholinergic transmission between young and the aged bladders. This is different to what was observed in a study by Yoshida et al. (2001), which measured the release

of neurotransmitters, ACh and ATP from isolated human detrusor tissue, and demonstrated a significant negative correlation ($r = -0.91$) between age and the amount of ACh released by electrical field stimulation (EFS), and a significant positive correlation ($r = 0.83$) between age and the amount of ATP released, suggesting that purinergic and cholinergic neurotransmission increases and decreases, respectively with age.

The effect of L-NNA on neurogenic responses was also examined but the responses were not enhanced in the presence of L-NNA in this study which is consistent with a previous study using rats that did not reveal any alteration of the contractile responses to EFS in normal tissues by L-NNA but significantly increased contractile responses in the bladders from the cyclophosphamide (CYP)-induced cystic rats (Vesela et al., 2012). Thus, it appears that NO does not influence contractile responses of detrusor muscle to efferent nerve stimulation in the healthy bladder or following cytotoxic drugs in the short-term (my study), but may in the longer term. A previous study supports this idea. The study evaluated the effects of nitric oxide synthase (NOS) on detrusor overactivity after removal of bladder outlet obstruction in rats. Rats were divided into a control group and an experimental group. The bladders in the experimental group were partially obstructed for 3 weeks. After removal of obstruction, the experimental group was subdivided into 'normalised' and 'overactive' groups, on the basis of cystometrogram results. Expression of neuronal, inducible, and endothelial NOS (nNOS, iNOS and eNOS, respectively) mRNA in bladders of each group was analysed by RT-PCR. Significantly decreased ($P < 0.05$) expression of nNOS and iNOS mRNA was demonstrated in the normalised group compared to the control group, and nNOS levels were increased ($P < 0.05$) in the overactive group compared to the normalised group. In addition, a significant increase ($P < 0.05$) in eNOS mRNA expression was shown in the normalised and overactive group compared to the control group. The findings of the study suggest that increases in nNOS and eNOS expression may be related to OAB conditions (Kim et al., 2008).

β -adrenoceptor-mediated relaxation

Pre-contractions induced by carbachol in all tissues from both young ($\approx 63\%$) and the aged ($\approx 62\%$) bladders were relaxed by isoprenaline. This finding is consistent with a previous study by Murakami et al. (2007) which also reported relaxation ($\approx 75\%$) of

carbachol pre-contracted pig tissues including intact bladder and detrusor muscle in response to isoprenaline. A study by Moro and his colleagues (2013) has also demonstrated relaxation and slowing of spontaneous phasic contraction of porcine urothelial/LP strips by isoprenaline.

The potency and maximum relaxation in intact bladder and detrusor muscle tissues were similar between young and the aged bladders. Similar observations were made in a previous study by Kolta et al. (1984) in which there was no difference in isoprenaline-induced relaxations of isolated whole bladders from rats aged between 7-month and 29-month. Also, another study reported no-aged related changes in contractile response of isolated whole rat bladder to isoprenaline (ages 5-7, 16-18 and 22-24 months) (Chun et al., 1989).

In contrast, isoprenaline was found to be more potent in urothelial/LP tissues from the aged pig bladders compared to young bladders. In addition, maximum relaxation in the urothelium/LP was also demonstrated to be greater in the aged bladders in comparison to young bladders. It can be suggested that the ability of urothelial/LP tissues to relax as well as sensitivity to β -adrenergic stimulation may increase with age.

Following doxorubicin treatment, the potency and maximum relaxation were similar to control incubated tissues in both young and the aged bladders, suggesting that the ability of tissues to relax in response to β -adrenergic stimulation are not affected by doxorubicin treatment.

Integrity of bladder morphology

Histological examination of cross-section of the bladder revealed that urothelial thickness of the aged bladders was significantly thinner than those of young bladders. Urothelial thinning was also reported in a study by Zhao et al. (2010) which found significantly decreased urothelial thickness in old (28-30 months) compared to young (4-6 months) rat bladders. There were also other age-related changes in anatomy of the bladder reported by the same study. These include a decrease in muscle mass and an increase in collagen deposition in the bladders of old rats compared to young rats. In addition, old rats had higher bladder weight than young rats. No studies have reported age-associated changes in urothelial thickness in human and it is also

unclear from the histological image taken from this study to determine whether decreased thickness of the urothelium is due to a change in cell number or volume. The finding of urothelial thinning with ageing could provide an explanation to why doxorubicin was found to be more cytotoxic in the aged bladders compared to young bladders in this study, potentially leading greater doxorubicin penetration into the deeper layer of the bladder, thus affecting not only the urothelium but also the detrusor muscle. In addition, the present study found no change in urothelial thickness in either young or aged bladders following doxorubicin treatment.

Conclusions

Here we demonstrated the effects of ageing on various function of the bladder. Contractile response to muscarinic stimulation were reduced, neurogenic responses were greater, mediator release from the urothelium/LP were greater, relaxation responses of the urothelium/LP were enhanced and lastly urothelial thickness was reduced in the aged bladders compared to young bladders.

It can be concluded that doxorubicin treatment may exerts its adverse effects in patients through enhancing ATP release from the urothelium/LP and increasing urothelial/LP contractile response to muscarinic stimulation in both young and the aged bladders. In the aged bladders, however, doxorubicin appears to have greater toxic effects on bladder function affecting deeper layer, detrusor muscle and this appears to be due to urothelial thinning observed in the aged bladders. Such toxic effects caused an increase in contractile activity of detrusor muscle to muscarinic stimulation as well as depression of neurogenic detrusor contractility (via damage on the efferent nerves) in which the former may also provide an explanation to the mechanism by which doxorubicin treatment may exert overactive activity in elderly patients following treatment. Whilst in young bladders, doxorubicin treatment may also contribute to overactivity of the bladder via increasing the release of neurotransmitters as observed by an enhanced neurogenic response following treatment.

Chapter 4 - The effects of mitomycin C and epirubicin on aged porcine bladder tissue

Introduction

The previous chapter demonstrated changes in tissue responses and mediator release with ageing and also doxorubicin pretreatment. Apart from doxorubicin, intravesical chemotherapeutic treatment with mitomycin C (MMC) and epirubicin are also widely practiced by clinicians to reduce progression and recurrence of bladder cancer. General introduction about these two cytotoxic drugs in addition to their detailed antitumor and cytotoxic mechanisms have been mentioned previously in Chapter 1. Thus, in this chapter, the following introduction for each agent will specifically focus on the efficacy and incidence of urinary adverse effects.

Intravesical mitomycin C (MMC)

A randomised clinical trial study by Trolley et al. (1996) involving 502 patients with newly diagnosed superficial bladder cancer has revealed the positive benefit of MMC to decrease the number of subsequent recurrences and increase the recurrence-free interval. In that trial, after resection of tumor, the patients were randomised into 1 of 3 treatment groups; no further treatment, 1 instillation of MMC at resection, and 1 instillation at resection and at 3-month interval for 1 year (total 5 instillations). A 40-mg dose of MMC in 40ml water was administered intravesically. The study found that 1 and 5 instillations of MMC resulted in decreased recurrence rates and increased recurrence-free interval compared to no treatment group and there was suggestive but not conclusive evidence that 5 instillations of MMC offered a slightly higher advantage over 1 instillation.

Shelley et al. (2003) conducted a meta analysis study and reported that 2% to 21% of patients developed cystitis, while allergy occurred in 7% to 16% of patients treated with intravesical MMC (Krege et al., 1996; Lamm et al., 1995; Rintala et al., 1991; Witjes et al., 1998; Witjes et al., 1996). Overall, approximately 30% of those patients receiving intravesical MMC were found to be developing local toxicities (dysuria, cystitis, frequency and haematuria). Also, in another study, contracted bladder was reported in three out of 125 patients treated with MMC (Malmstrom et al., 1999).

Mangiarotti et al. (2008) reported that 11 out of 46 patients with low grade recurrent superficial bladder cancer developed grade 1-2 toxicity (mild or moderate cystitis in

6 patients and mild or moderate hypersensitivity reactions in 5) and grade 3 toxicity in 11 patients (severe cystitis in 4, gross haematuria in 2 and severe hypersensitivity in 5 cases) after MMC treatment. Intravesical treatment had to be terminated in 11 patients.

Also, increased urinary frequency occurred in 40.7% of patients (31 of the 76 patients) with Ta, T1 and TIS transitional cell carcinoma of the bladder after receiving eight weekly instillations of intravesical MMC (Heney et al., 1988).

A study by Au et al. (2001) randomised 217 patients with histologically proven transitional cell carcinoma and at high risk for recurrence into two groups to test whether enhancing the concentration of MMC in urine would improve its efficacy. Patients in the optimised-treatment group (N=119) received a 40-mg dose of MMC, pharmacokinetic manipulations to increase drug concentration by decreasing urine volume, and urine alkalinisation to stabilize the drug. Patients in the standard-treatment group (N=111) received a 20-mg dose without pharmacokinetic manipulations or urine alkalinisation. Patients in both groups received six weekly treatments with intravesical MMC dissolved in 20 mL of sterile water for 2 hour. In the study, patients in the optimised group showed a longer median time to recurrence (29.1 months) and a greater recurrence-free fraction (41%) at 5 years than patients in the standard group (11.8 months and 25%, respectively). Minimal differences in toxicity were observed in the two treatment groups. The incidence of urinary frequency/urgency in the optimised and standard groups were 31%/26% and 24%/22%, respectively. Cystitis occurred in 23% of patients in the optimised group and in 16% of patients in standard group. 18% and 19% of patients experienced fatigue in the optimised and normal groups, respectively. Dysuria was the only toxic effect that occurred more frequently in the optimised group (33%) compared to the standard group (18%), but did not result in more frequent termination of treatment (1.8% and 1.9%, respectively). Grade 3 dysuria (painful urination not controlled by pyridium) occurred in four patients in the optimised group but in no patients in the standard group. Twelve patients who complained of dysuria or cystitis (two in the standard group and 10 in the optimised group) were treated with antibiotics for possible urinary-tract infection. The study suggests that a higher dose of intravesical MMC (2mg/ml) compared to normal dose (1mg/ml) can enhance efficacy of the drug without seeing a significant increase in bladder toxicity.

Intravesical epirubicin

A study by Sengor et al. (1996) which included 45 patients with Ta-T1 superficial bladder cancer has found that intravesical epirubicin therapy is effective in the prophylaxis of superficial bladder cancer, reducing the recurrence rate and prolonging time to recurrence. Fourteen patients who had transurethral resection (TUR) of the tumour alone were assigned to the control group and the rest of the patients received 8 weekly instillations of intravesical epirubicin (50mg diluted in 50ml saline) two weeks after complete TUR of the tumour (the epirubicin group). The study found a higher recurrence rate in the control group (64% versus 32%) which was not statistically significant, but importantly also demonstrated that tumour-free intervals in patients with recurrent disease were significantly longer in the epirubicin group.

In a report by Ali-el-Dein et al. (1997), epirubicin has been demonstrated to be more efficacious and less toxic than doxorubicin when used as an intravesical agent. In that study, a total of 253 patients with superficial bladder cancer were randomly allocated to 4 study arms:

- (1) 50mg epirubicin after TUR
- (2) 80mg epirubicin after TUR
- (3) 50mg doxorubicin after TUR
- (4) TUR alone

Instillation was repeated weekly for 8 weeks, and monthly thereafter, to complete 1 year of treatment. The study found that rates of recurrence were significantly lower in the chemotherapy groups than in controls ($P<0.001$) and lower in the epirubicin groups than in the doxorubicin group ($P<0.05$). Recurrence rates were 25%, 18%, 37% and 66% in arms 1 to 4, respectively. Also, mean interval to first recurrence was 16, 15.4, 18.9 and 6.3 months, respectively, with a significant difference ($P<0.05$) between the chemotherapy and control groups. Local toxicity developed in 10 (16%), 16 (24%) and 25 (42%) patients in chemotherapy arms 1 to 3, respectively. Significantly lower toxicity rates in arms 1 and 2 than in 3 ($P<0.01$) was reported, and a marginal insignificant difference was demonstrated between low and high dose epirubicin ($P=0.3$). It appears that intravesical treatment with epirubicin is more efficacious on bladder cancer and also less cytotoxic than intravesical doxorubicin.

However, the results of the study suggest that the efficacy of epirubicin is not concentration-dependent.

In another study by Hendricksen et al. (2008), the effects of either an early instillation or maintenance instillations of adjuvant intravesical epirubicin (50mg in 50ml NaCl for 1 hour), as compared to the epirubicin standard treatment schedule only were evaluated, in patients with non-muscle invasive bladder cancer. The patients were randomised into 3 groups:

- (1) 4 weekly and 5 monthly instillations (standard schedule)
- (2) The same schedule as group 1, but with an additional instillation less than 48 hour after TUR
- (3) The same scheme as group 1, but with additional instillations at 9 and 12 months (maintenance schedule).

Standard follow-up was 5 year and consisted of cystoscopy, cytology, and registration of adverse events. The study showed that out of a total of 731 patients, 44%, 43%, and 45% of the patients, respectively, in groups 1, 2, and 3 were recurrence free, and 90%, 88%, and 88% of the patients, respectively, were progression free. Also, 23%, 30%, 15% and 9% of patients who received at least one instillation of epirubicin developed bacterial cystitis, chemical cystitis, haematuria and other local side effects, respectively. While side effects were not significantly different between treatment groups, haematuria occurred significantly more in the immediate instillation group (2) (19%) compared to group 1 (14%) and group 3 (11%). Thirteen percent of the patients reported systemic side effects which included tiredness, fever, nausea, headache, and abdominal pain. Seven percent of the patients discontinued the instillations due to local (6%) or systemic (1%) side effects.

It can be seen that both MMC and epirubicin can effectively reduce recurrence of bladder cancer if used intravesically after TUR compared to TUR alone. However, despite the advantage of direct exposure of cytotoxic drugs to tumours with minimal systemic introduction, various bladder toxicities occur in patients. Both cytotoxic agents commonly reported chemical cystitis such as increased urinary urgency and frequency, and dysuria.

Aims

Since, bladder cancer is a disease of senior adults and the differences in bladder functions were observed between young and aged pigs, bladders from the aged pigs were used in this study. Thus, the aim of this study was to investigate the effects of two other commonly used intravesical chemotherapeutic agents MMC and epirubicin on normal bladder functions including mediator release, integrity of bladder morphology, tissue responsiveness and sensitivity, and neurogenic responses.

Materials and Methods

(Refer to “Porcine tissue experiments” and “Assays for urothelial mediators, NO and inflammatory cytokines” sections of Chapter 2 for full detail)

Full thickness sheets of bladder wall from aged pigs (2 to 3 years old; approx. 250kg) were set up in treatment chambers and therapeutic concentration of either MMC (2mg/ml) or epirubicin (1mg/ml) was exposed to the urothelial surface for 2 hour or 1 hour, respectively. Both cytotoxic drugs were diluted in fresh Krebs-bicarbonate solutions.

Following pretreatment with either MMC or epirubicin, tissue responses, urothelial/LP mediator release, and bladder morphology were evaluated. The release of mediators was measured using commercially available kits.

Results

Contractile responses to carbachol

All tissues contracted to carbachol again as it was already demonstrated in doxorubicin study previously (Figure 3.1). The potency (pEC_{50} values) of all tissues in each control group was similar to each other, but the maximum responses varied among each control groups. While the maximum responses of intact bladder and detrusor muscle tissues in controls from epirubicin and doxorubicin studies were similar, the maximum response of these tissues in controls from MMC study was significantly smaller compared to controls from other two studies (Table 4.1 and Table 3.1 from doxorubicin study). Also, the maximum responses of urothelial/LP tissues in controls from MMC and doxorubicin studies were similar, but the maximum response of this tissue in control from epirubicin study was significantly smaller compared to controls from other two studies (Table 4.1 and Table 3.1 from doxorubicin study).

MMC pretreatment did not affect the maximum contraction of intact bladder tissues to carbachol, but the maximum contractions of pretreated detrusor muscle and urothelial/LP tissues to carbachol were significantly reduced ($P<0.05$ and $P<0.01$, respectively) by 15.9% and 23.6%, respectively, compared to control tissues (Figure 4.1; Table 4.1).

Epirubicin pretreatment did not affect any of the tissue responses to carbachol except the maximum contraction of detrusor muscle was significantly reduced ($P<0.05$) by 26.7% compared to control tissues following treatment (Figure 4.1; Table 4.1).

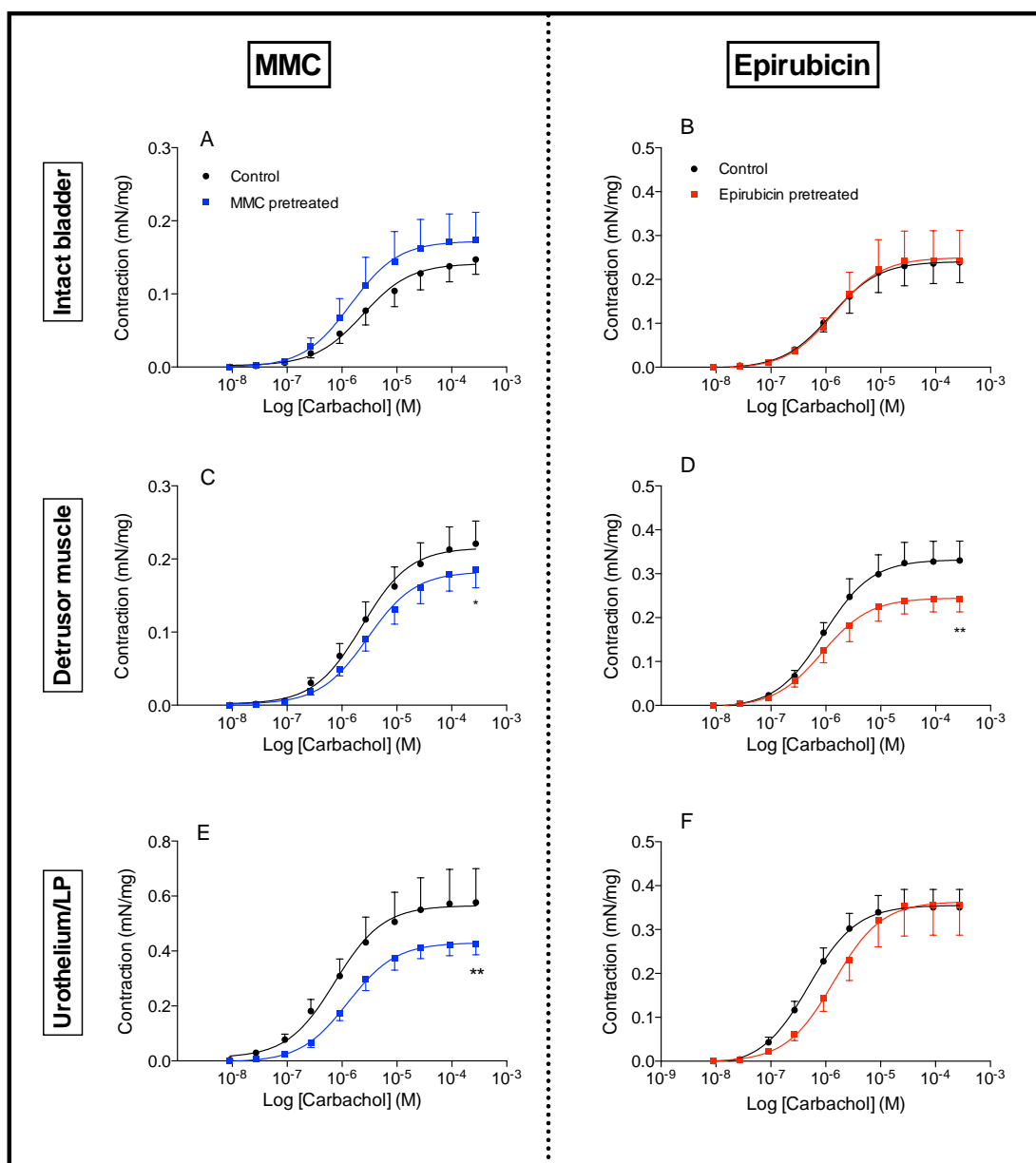


Figure 4.1: Cumulative concentration-response curves to carbachol of tissues from control and pretreated bladders by MMC or epirubicin. Responses of intact bladder [A & B], detrusor muscle [C & D] and urothelium/LP [E & F]. Data are expressed as means \pm SEM. * $P < 0.05$ and ** $P < 0.01$ compared to maximum response of control tissue.

Table 4.1: Potency (pEC₅₀) values and maximum responses (mN/mg tissue) to carbachol of intact bladder strips, detrusor muscle strips and strips of urothelium/LP. Data are expressed as means \pm SEM.

MMC						
Tissue	Control (untreated) bladders			Pretreated bladders		
	Potency	Maximum	N	Potency	Maximum	N
Intact bladder	5.62 \pm 0.18	0.14 \pm 0.001 ^b	8	5.84 \pm 0.26	0.17 \pm 0.02	8
Detrusor muscle	5.64 \pm 0.15	0.22 \pm 0.01 ^c	8	5.53 \pm 0.14	0.18 \pm 0.01 ^a	8
Urothelium/LP	6.13 \pm 0.24	0.57 \pm 0.04	7	5.89 \pm 0.11	0.43 \pm 0.02 ^a	8
Epirubicin						
Intact bladder	5.90 \pm 0.20	0.24 \pm 0.02	7	5.85 \pm 0.29	0.25 \pm 0.03	7
Detrusor muscle	6.04 \pm 0.15	0.33 \pm 0.02	6	6.07 \pm 0.16	0.24 \pm 0.01 ^a	6
Urothelium/LP	6.31 \pm 0.13	0.36 \pm 0.02 ^d	8	5.85 \pm 0.20	0.36 \pm 0.03	8

N number of bladders examined

^a P<0.05 and P<0.01 compared to corresponding control tissue.

^b P<0.001 compared to corresponding control tissues from epirubicin and doxorubicin (aged bladders from Table 3.1).

^c P<0.01 compared to corresponding control tissues from epirubicin and doxorubicin (aged bladders from Table 3.1).

^d P<0.001 compared to corresponding control tissues from MMC and doxorubicin (aged bladders from Table 3.1).

Relaxation responses to isoprenaline

All tissues relaxed to isoprenaline (Figure 4.2) and the potency (pEC_{50}) values were similar on all three tissues from both MMC and epirubicin studies (Table 4.2).

For MMC pretreated bladders, there was no difference in the maximum relaxation to isoprenaline (expressed as percentage of pre-contraction to carbachol) between control and pretreated intact bladder tissues, and also between control and pretreated urothelial/LP tissues. However, the maximum relaxation of pretreated detrusor muscle tissues to isoprenaline was significantly greater ($P < 0.05$) than control detrusor muscle tissues (Figure 4.2; Table 4.2).

Following epirubicin pretreatment, the maximum relaxation of all three tissues to isoprenaline was similar to control tissues (Figure 4.2; Table 4.2).

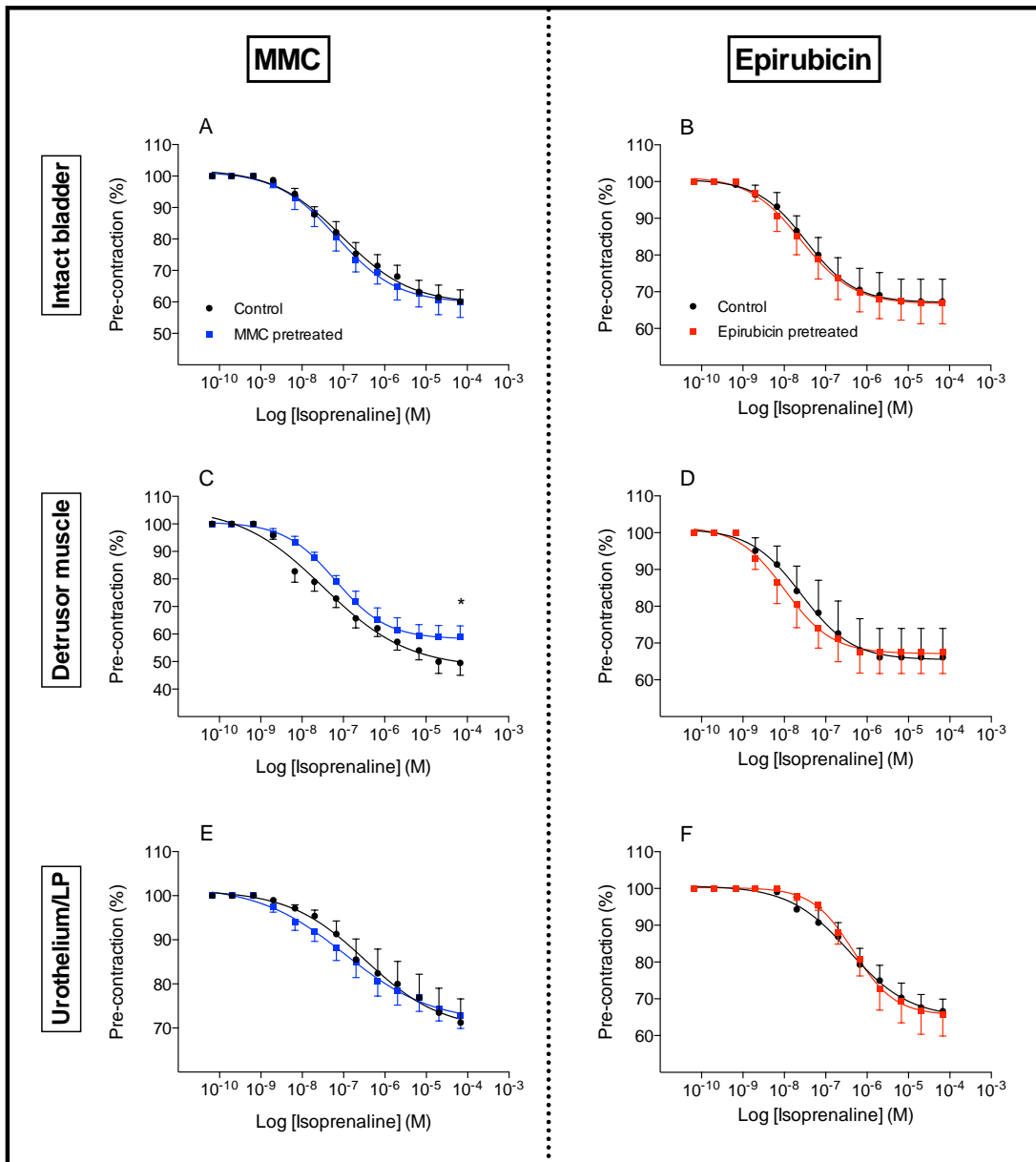


Figure 4.2: Cumulative concentration-response curves to isoprenaline in tissues from control and pretreated bladders by MMC or epirubicin. Responses expressed as a percentage of the pre-contraction to carbachol (27.4 μ M). Responses of intact bladder [A & B], detrusor muscle [C & D] and urothelium/LP [E & F]. Data are expressed as means \pm SEM. *P<0.05 compared to control tissue.

Table 4.2: Potency (pEC₅₀) values and maximum relaxation (%) to isoprenaline of intact bladder strips, detrusor muscle strips and strips of urothelium/LP. Data are expressed as means \pm SEM.

MMC						
Tissue	Control (untreated) bladders			Pretreated bladders		
	Potency	Maximum	N	Potency	Maximum	N
Intact bladder	7.04 \pm 0.19	59.42 \pm 2.89	8	7.16 \pm 0.20	59.70 \pm 2.91	8
Detrusor muscle	7.48 \pm 0.21	47.68 \pm 3.68	8	7.19 \pm 0.14	58.16 \pm 2.08 ^a	8
Urothelium/LP	6.48 \pm 0.46	69.60 \pm 6.23	7	6.97 \pm 0.33	71.25 \pm 3.92	6
Epirubicin						
Intact bladder	7.48 \pm 0.25	67.08 \pm 2.66	6	7.59 \pm 0.26	66.66 \pm 2.63	6
Detrusor muscle	7.59 \pm 0.35	65.47 \pm 3.71	5	7.99 \pm 0.28	67.13 \pm 2.39	5
Urothelium/LP	6.43 \pm 0.23	64.93 \pm 3.61	6	6.33 \pm 0.18	65.57 \pm 3.01	5

N number of bladders examined

^aP<0.05 compared to control denuded detrusor.

Detrusor responses to electrical field stimulation

The detrusor responses to electrical field stimulation (EFS) increased with increasing stimulation frequency and the responses were depressed in the MMC pretreated tissues compared to control tissues with a statistically significant depression ($P < 0.05$) at the lower stimulation frequencies of 1Hz and 5Hz (Figure 4.3a). The responses of both control and epirubicin pretreated detrusor strips to EFS also increased with increasing stimulation frequency, however there was no difference in the responses between the control and the epirubicin pretreated tissues (Figure 4.3b).

The detrusor muscle responses to EFS did not change in the presence of the nitric oxide synthase inhibitor (L-NNA, 100 μ M) when compared to the normal responses without L-NNA (Figure 4.3c, d, e and f).

The effect of atropine on detrusor responses was also examined. A cholinergic neurotransmission was observed in all tissues (control, MMC pretreated & epirubicin pretreated tissues), however there was no difference in the cholinergic component (atropine sensitive) between control and pretreated tissues (Figure 4.3c, d, e and f; Table 4.3).

In addition, desensitisation of P_2 -purinoceptor with α,β -mATP had no effect on responses to EFS in either control or pretreated tissues (Figure 4.3c, d, e and f).

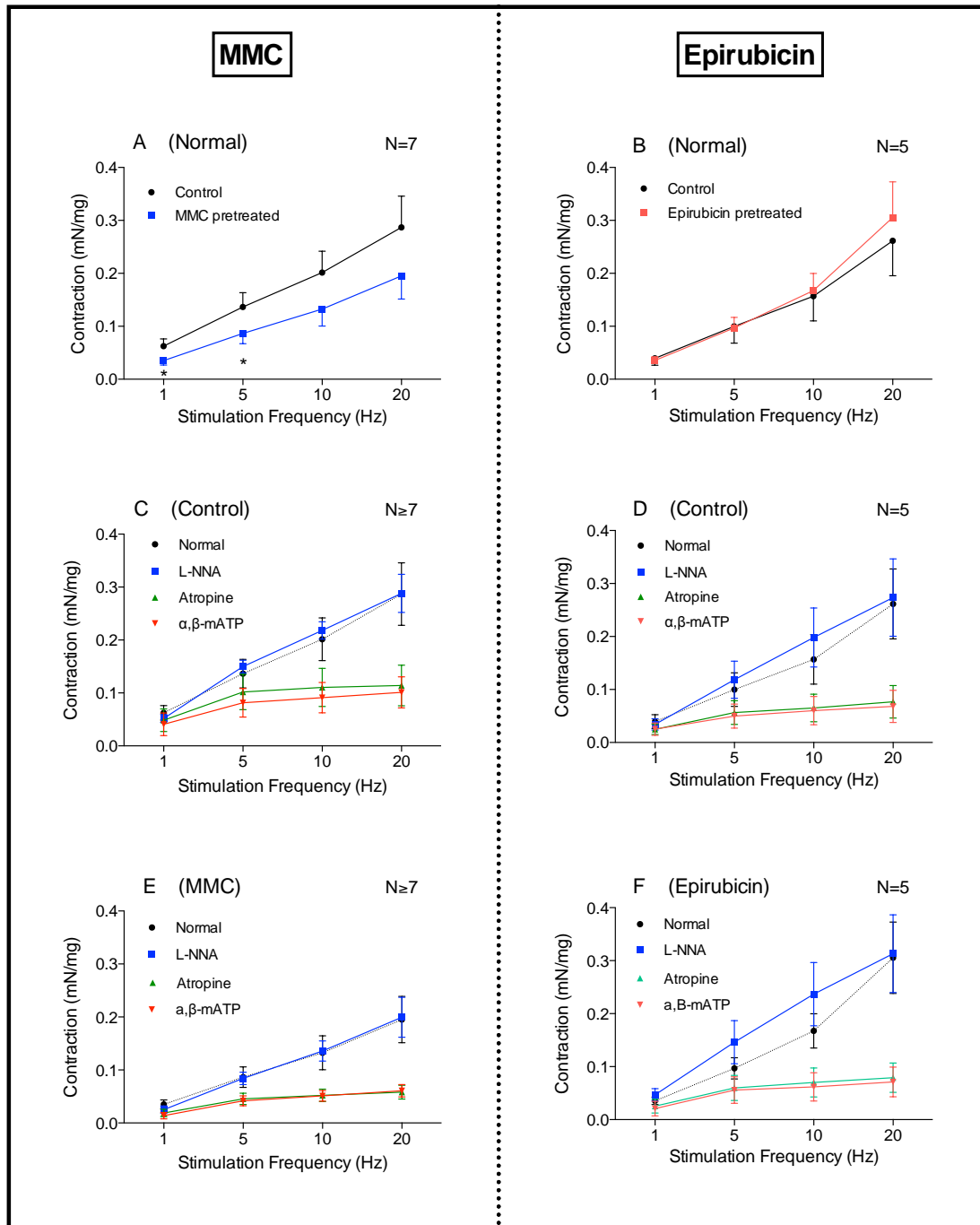


Figure 4.3: Detrusor muscle responses to electrical field stimulation (EFS) in tissues from control bladders and bladders pretreated with MMC [A] or epirubicin [B]. Effect of L-NNA, atropine and α,β -mATP on tissues from control [C & D] bladders and bladders pretreated with MMC [E] or epirubicin [F]. Data are expressed as means \pm SEM. * $P < 0.05$ compared to control incubated tissues.

Table 4.3: Reduction (%) in detrusor response to EFS in the presence of atropine (1 μ M). Data are expressed as means \pm SEM.

MMC				
Frequency (Hz)	Control (untreated) bladders		Pretreated bladders	
	Reduction (%)	N	Reduction (%)	N
1	64.8 \pm 12.2	8	50.1 \pm 20.8	8
5	49.2 \pm 16.6	8	47.0 \pm 13.3	8
10	59.7 \pm 14.9	8	53.1 \pm 11.4	8
20	73.0 \pm 9.9	8	65.0 \pm 10.3	8
<i>Average</i>	<i>61.68\pm13.4</i>		<i>53.8\pm13.95</i>	
Epirubicin				
1	63.7 \pm 16.3	5	60.6 \pm 15.7	5
5	56.0 \pm 14.9	5	61.7 \pm 9.4	5
10	68.9 \pm 9.8	5	64.1 \pm 8.5	5
20	77.9 \pm 6.1	5	76.5 \pm 4.2	5
<i>Average</i>	<i>66.63\pm11.78</i>		<i>65.73\pm9.45</i>	

N number of bladders examined

Urothelial mediators

Isolated strips of urothelium/LP from the control incubated bladders of the MMC study released all three mediators, but the basal release of ACh was on average 270-fold greater than that of ATP, and the basal release of ATP was approximately 2-fold greater than that of PGE₂. Stretching the tissues produced on average a 3-fold increase in ATP release, but had no effect on either ACh or PGE₂ release (Figure 4.4). In tissues from bladders pretreated with MMC, the basal release of ATP and ACh was similar to control tissues at all time points. However, the basal release of PGE₂ in pretreated tissues was significantly ($P<0.05$) increased (2-fold) at 7 minutes compared to tissues from control bladders. Unlike tissues from control bladders, stretching MMC-pretreated tissues did not affect any mediator release. The stretch-induced release of PGE₂ from pretreated tissues was similar to control tissues at all times. Also, the stretch-induced ACh release was similar to the control at all time points except at 3 minutes where a significant increase was observed. However, the stretch-induced release of ATP in MMC pretreated tissues was significantly ($P<0.05$ and $P<0.01$) reduced (on average 80%) at 2, 3 and 7 minutes compared to control tissues (Figure 4.4).

Urothelial/LP strips from the control incubated bladders for the epirubicin experiment released all three mediators but the basal release of ACh was on average 1470-fold greater than that of ATP, and the basal release of ATP was approximately 2-fold greater than that of PGE₂. In this series of experiments, stretching the control tissues did not increase release of ACh and PGE₂. It appeared to produce an increase in ATP release, but the difference was not statistically significant (Figure 4.5). In tissues from epirubicin pretreated bladders, the basal release of ATP, ACh and PGE₂ was similar to control tissues at all time points, except a significant increase was observed at 7 minutes for basal release of ACh compared to control. Stretching urothelial/LP tissues from epirubicin pretreated bladders produced on average a 2-fold increase ($P<0.05$) in PGE₂ release, but had no effect on either ATP or ACh release. The stretch-induced release of ACh and PGE₂ from pretreated tissues was similar to control tissues at all time points. However, the stretch-induced release of ATP in epirubicin pretreated tissues was significantly ($P<0.05$) reduced (halved) at 7 minutes compared to control tissues (Figure 4.5).

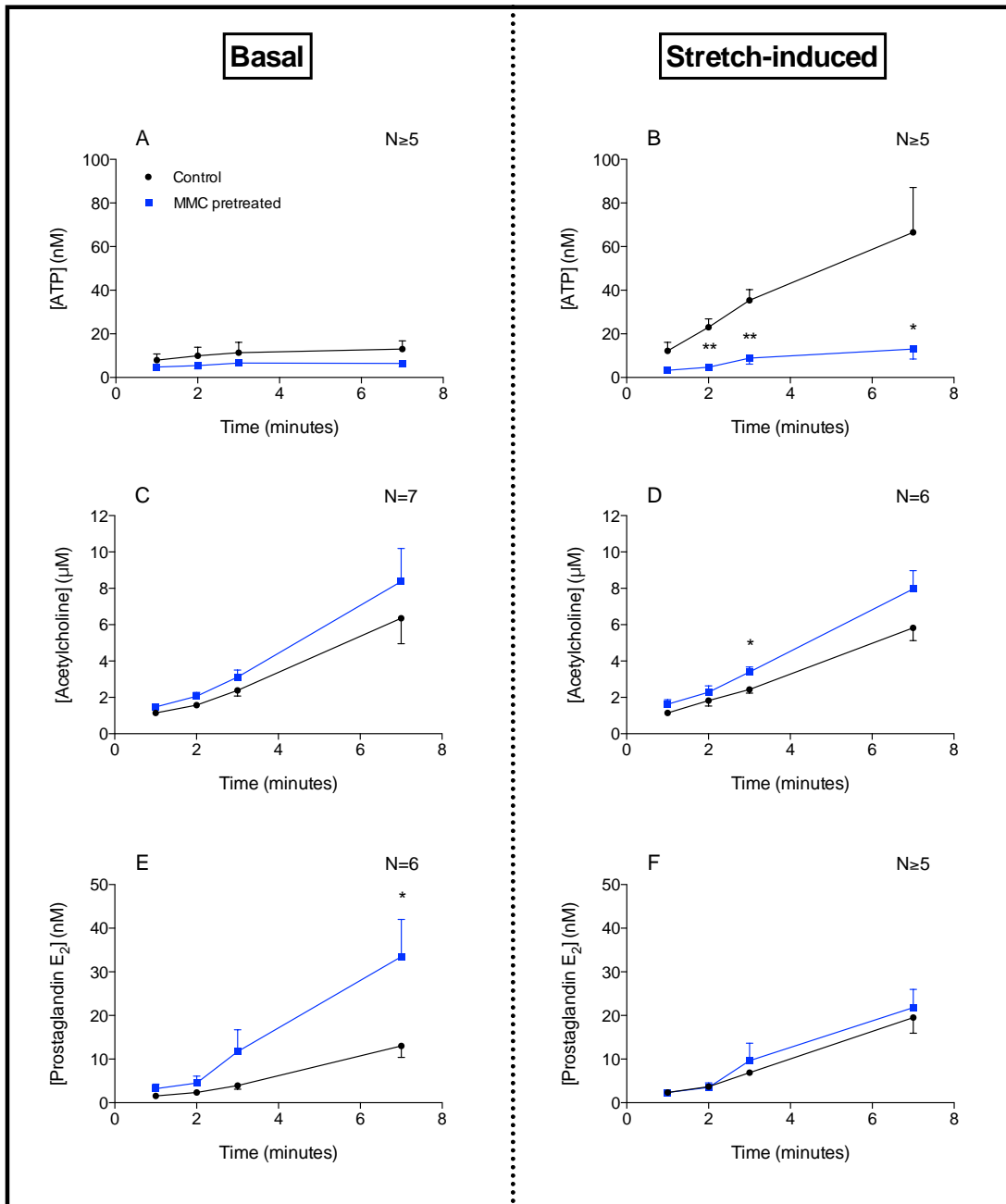


Figure 4.4: Mediator release from urothelial/LP tissues. Effects of MMC pretreatment on ATP [A], acetylcholine [C] and prostaglandin E₂ [E] concentrations after 7 minute incubation, and ATP [B], acetylcholine [D] and prostaglandin E₂ [F] concentrations after 7 minute incubation that included stretching over the initial 2 minutes. Data are expressed as means ± SEM. *P<0.05 and **P<0.01 compared to control incubated tissues.

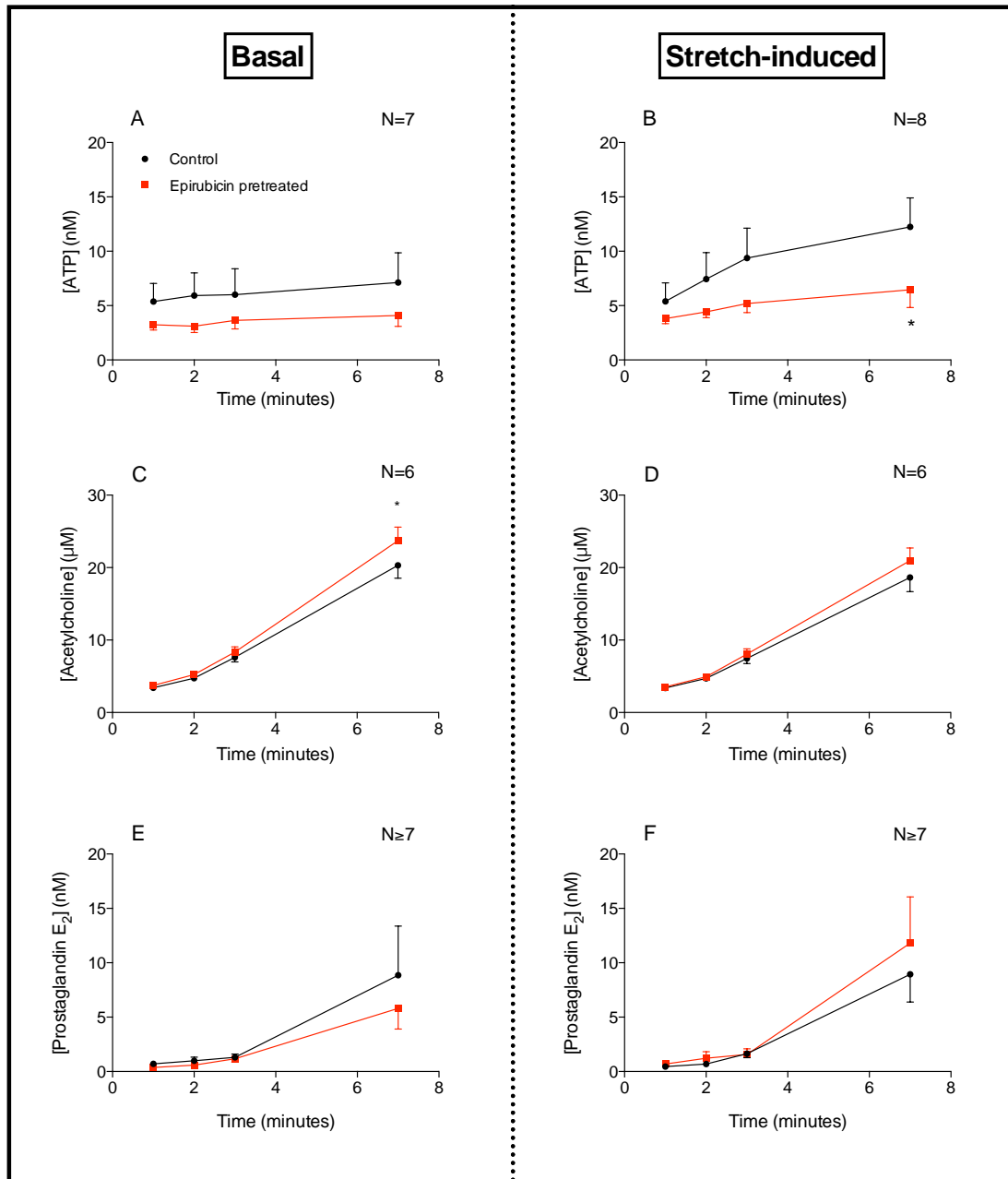


Figure 4.5: Mediator release from urothelial/LP tissues. Effects of epirubicin pretreatment on ATP [A], acetylcholine [C] and prostaglandin E₂ [E] concentrations after 7 minute incubation, and ATP [B], acetylcholine [D] and prostaglandin E₂ [F] concentrations after 7 minute incubation that included stretching over the initial 2 minutes. Data are expressed as means ± SEM. *P<0.05 compared to control incubated tissues.

Inhibition of detrusor contraction by the urothelium

The presence of an intact urothelium/LP on strips of detrusor muscle inhibited contractions to carbachol in control tissues but not in MMC pretreated bladders (Figure 4.6). In control tissues the potency of carbachol was similar in intact bladder and detrusor muscle tissues, but the maximum responses were significantly ($P<0.001$) depressed ($27\pm12\%$) in intact bladder compared to detrusor muscle tissues. However, different results were obtained in tissues from MMC pretreated bladders, with both the potency of carbachol and the maximum responses being similar in intact bladder and detrusor muscle tissues (Table 4.1).

Similarly, the presence of an intact urothelium/LP on strips of detrusor muscle inhibited contractions to carbachol in control tissues but not in epirubicin pretreated bladders (Figure 4.6). The potency of carbachol in control tissues was similar in intact bladder and detrusor muscle tissues, but the maximum responses were again significantly ($P<0.01$) depressed ($33\pm14\%$) in intact bladder compared to detrusor muscle tissues. In contrast, both the potency of carbachol and the maximum responses of epirubicin pretreated bladders were similar in intact bladder and detrusor muscle tissues (Table 4.1).

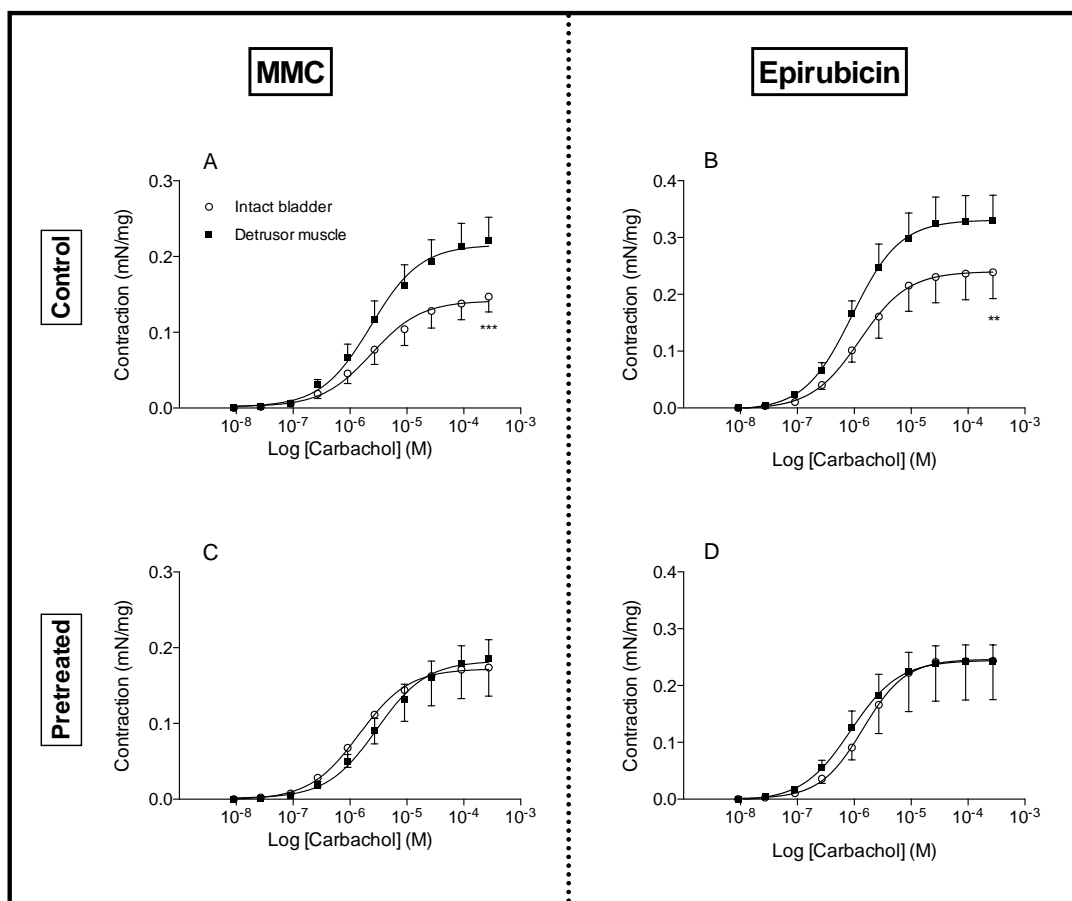


Figure 4.6: Inhibitory effects of the urothelium/LP on detrusor responses to carbachol in intact bladder and detrusor muscle tissues from control [A & B], MMC [C] and epirubicin [D] pretreated bladders. Data are expressed as means \pm SEM. ** $P < 0.01$ and *** $P < 0.001$ compared to maximum response of detrusor muscle.

Bladder histology

As in the doxorubicin study, tissue sections from untreated bladder demonstrated typical histological features such as the urothelium and lamina propria thrown into many folds and also a layer of detrusor smooth muscle underneath these layers (Figure 3.7 and 3.8).

Histologically, the bladder structure was affected by both MMC and epirubicin pretreatment. Urothelial thickness of MMC pretreated bladders ($22.93 \pm 2.91 \mu\text{m}$, N=4) was significantly ($P < 0.05$) thinner compared to control incubated bladders ($32.77 \pm 2.05 \mu\text{m}$, N=4) (Figure 3.7). In addition, urothelial thickness of epirubicin pretreated bladders ($24.79 \pm 3.08 \mu\text{m}$, N=4) was also significantly ($P < 0.05$) thinner compared to control ($40.16 \pm 5.08 \mu\text{m}$, N=4) (Figure 3.8).

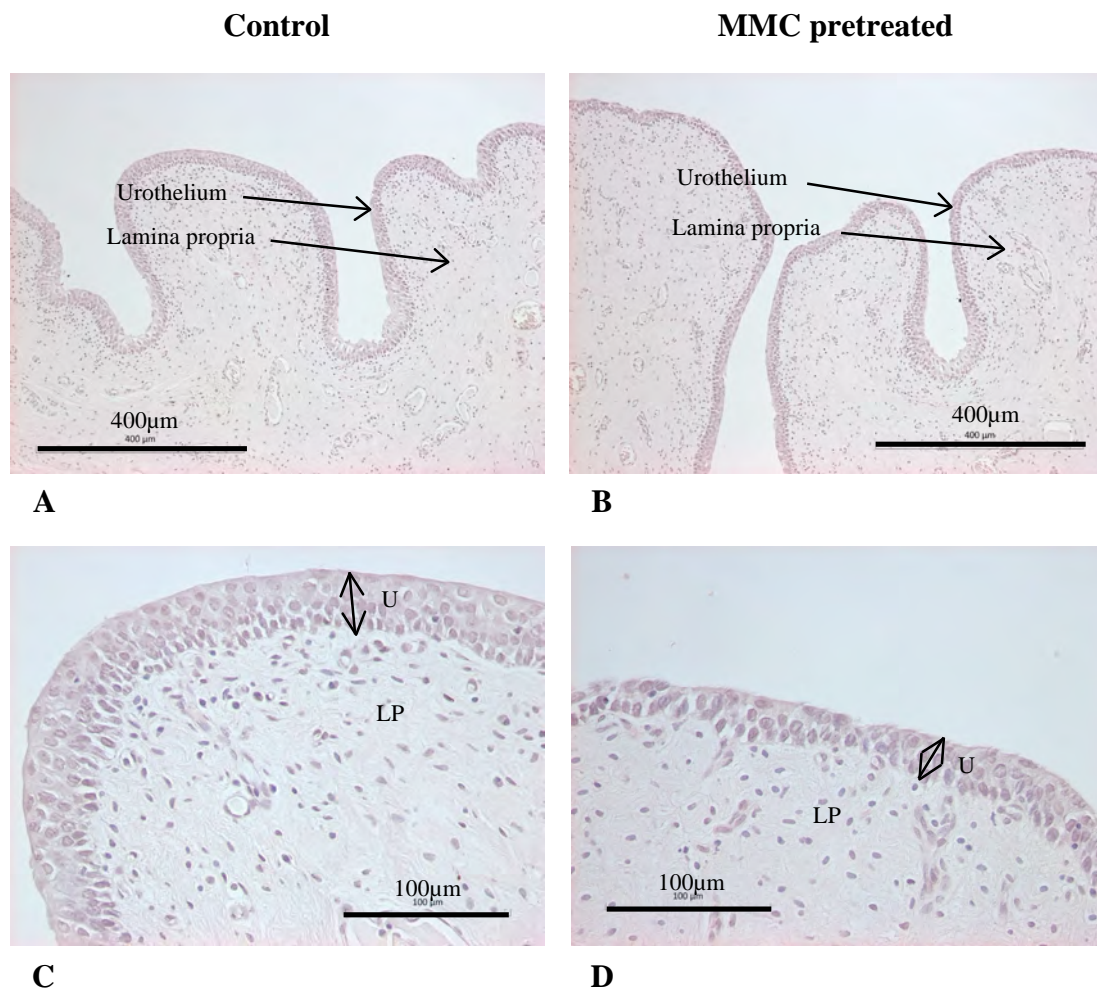


Figure 4.7: Haematological examination of bladder section. Haematoxylin and eosin (H and E) staining of control [A & C], and MMC pretreated [B & D] bladders. H and E staining at x10 [A & B] and x40 [C & D] showing the urothelium (*U*) and lamina propria (*LP*).

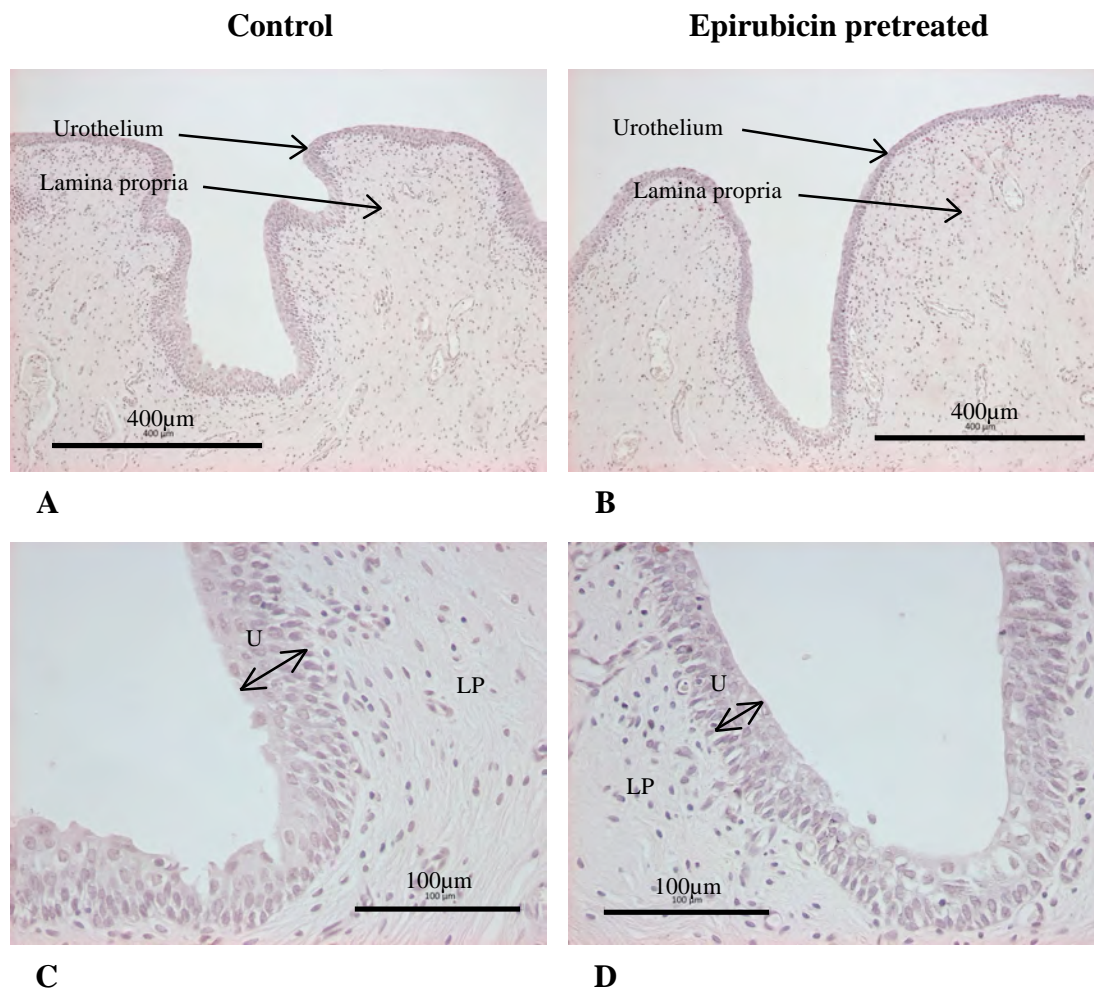


Figure 4.8: Haematological examination of bladder section. Haematoxylin and eosin (H and E) staining of control [A & C], and epirubicin pretreated [B & D] bladders. H and E staining at x10 [A & B] and x40 [C & D] showing the urothelium (U) and lamina propria (LP).

Discussion

Tissue responses

As in the doxorubicin study, in all tissues carbachol induced contraction. These contractions, when expressed relative to weight (mN/mg), were greater in urothelial/LP strips compared to those produced by detrusor muscle and intact bladder strips, which is consistent with a report by Sadananda et al. (2008).

Following pretreatment with MMC and epirubicin, the potency of carbachol was unaltered, but the maximum responses of detrusor muscle were significantly decreased compared to control tissues. Decreased detrusor contractility is generally associated with under activity of the bladder and the finding is surprising since intravesical MMC and epirubicin treatment is characterised by increased urinary urgency and frequency (Duque and Loughlin, 2000; Hendricksen et al., 2008; Kim and Lee, 1989; Onrust et al., 1999). Thus, the decreased detrusor contractility observed in this study is unlikely to explain the increased urinary urgency and frequency reported in patients following intravesical epirubicin treatment, but it may indicate direct muscle damage by MMC and epirubicin toxicity.

In addition, the neurogenic detrusor contractions elicited by nerve depolarisation during EFS was not affected by epirubicin, but was significantly depressed by MMC. The decrease in detrusor neurogenic responses by MMC was expected since the muscle responses to carbachol were also depressed after treatment. Similar to the observations made in the doxorubicin study, neither L-NNA nor α,β -mATP affected neurogenic responses but the responses were reduced in the presence of atropine, indicating a cholinergic component to detrusor contraction of porcine which was not affected by pretreatment with either MMC or epirubicin (discussed in detail in doxorubicin study).

Since, the decrease in neurogenic response (32%) was significantly greater than the decrease in muscle response (18%), it can be postulated that efferent nerves in detrusor muscle in addition to the detrusor muscle itself are potentially damaged by the toxic effect of MMC. The decreased detrusor contractility to exogenous agonist and to nerve stimulation following MMC treatment may possibly result from a maintained tonic contraction of the detrusor. Such a persistent contracted bladder has

been reported to occur in patients following intravesical MMC treatment (Kim and Lee, 1989).

Furthermore, the maximum responses of urothelial/LP tissues were also significantly decreased following MMC treatment and it is currently unclear what relevance the depressed urothelial/LP contractility may have to the development of OAB (see discussion Chapter 3).

The non-selective β -AR agonist isoprenaline induced relaxation in all tissues and these responses were not affected by pretreatment with epirubicin. However, while the potency of isoprenaline was unaffected by MMC, the maximum relaxation of detrusor muscle to isoprenaline was significantly reduced. This may also be due to a toxic effect of MMC on detrusor muscle and was evident by both a decreased detrusor contractility to exogenous agonist and to nerve stimulation. Activation of β -ARs is involved in relaxation of the bladder induced by sympathetic nerve activation during the filling phase. β -ARs are classified into three different subtypes, β_1 -, β_2 - and β_3 -ARs (Igawa et al., 2010), and the functional involvement of β -AR subtypes in bladder relaxation has been demonstrated to be species dependent (Morita et al., 2000; Takeda et al., 2003; Yamazaki et al., 1998). There is evidence suggesting that the relaxation induced by β -AR agonists in detrusor muscle may be mediated mainly via β_2 -AR and possibly β_3 -AR in pigs (Yamanishi et al., 2002b). In the human detrusor, a study by Igawa et al. (1998) has shown that the relaxation induced by adrenergic stimulation is mediated mainly by β_3 -AR, rather than β_1 - or β_2 -AR, since a non-selective β -AR antagonist bupranolol that is known to have antagonistic action only on β_1 - and β_2 -AR at a low concentration (10nM) did not inhibit isoprenaline-induced relaxation but at higher concentrations (0.1-10 μ M), which is known to also affect the β_3 -AR, the drug caused a rightward shift of the concentration-relaxation curve for isoprenaline in a dose-dependent manner. In addition, while neither a β_1 -AR agonist dobutamine nor a β_2 -AR agonist procaterol produced significant relaxation in human detrusor (Igawa et al., 1998; Igawa et al., 1999), the selective β_3 -AR agonists including BLR37,344, CL316,243, and CGP12,177A all produced concentration-dependent relaxation (Igawa et al., 1999; Takeda et al., 1999). Thus, an oral β_3 -AR agonist such as mirabegron is the newest option for treatment of OAB. In a two 12-week, randomised, double-blind, placebo-controlled Phase 3 studies, mirabegron has been reported to significantly reduce the number of incontinence

episodes and also micturitions per 24 hours were reduced from baseline values (Bridgeman et al., 2013). Several animal studies have revealed the *in vivo* function of β -AR agonists on bladder function. In a study by H. Takeda et al. (2000) using urethane-anaesthetised rats, isoprenaline, procaterol, and CL316,243 dose-dependently decreased intravesical pressure under isovolumetric conditions, and in cystometry experiments, CL316,243 significantly prolonged the micturition interval and increased bladder capacity. A further study by H. Takeda et al (2003) demonstrated reduced intravesical pressure in urethane-anaesthetised rats by both procaterol and CL316,243. In addition, whilst intravenous administration of procaterol did not affect voiding pressure relative to vehicle and had little effect on bladder capacity in conscious, unrestrained rats, CL316,243 had no effect on bladder capacity but reduced voiding pressure (Kaidoh et al., 2002).

Compliance is defined as the increase in pressure per unit increase in volume. Healthy bladder is very compliant and may be filled with large volumes with little increase in detrusor pressure (Sand and Ostergard, 1995), but a reduced maximum bladder capacity has been reported in patients following MMC treatment (Kim and Lee, 1989). Michielsen et al. (2005) has also reported decreased maximum volume and compliance of the bladder in mice treated with MMC. This decreased bladder compliance can result in greater pressure increases during filling. Therefore, the finding of reduced detrusor relaxation to β -AR stimulation observed in this study may provide a mechanism by which MMC could cause decreased bladder capacity in patients. Furthermore, decreased maximum bladder volume caused by low bladder compliance may be correlated to overactivity of the bladder (increased urinary urgency and frequency) reported in patients following MMC treatment because a decreased bladder volume would result in the bladder needing to be voided more frequently and thus urge to urinate will also be subsequently increased.

Integrity of bladder morphology

The effects of chemotherapeutic agents on urothelial integrity are unknown, but it was found that doxorubicin pretreatment does not affect urothelial thickness (Chapter 3). However, histological examination of the bladder sections that received MMC and epirubicin pretreatment, revealed that urothelial thickness was significantly thinner compared to those of control bladder sections, suggesting that these two

agents may cause an urothelial thinning in patients after intravesical treatment. Reduced urothelial thickness may indicate that the protective components of the urothelium such as uroplakins, tight junctions and GAGs are damaged. This can potentially compromise the protective function of the urothelium, which in a healthy bladder effectively prevents penetration of harmful solutions into the layers underneath the urothelium. The loss of barrier function may lead to increased susceptibility of chemotherapeutic agents to be absorbed by the urothelium itself as well as deeper layers of the bladder such lamina propria and detrusor muscle. This finding may explain why contractile responses of the urothelium/LP and the ability of detrusor muscle to relax were reduced by MMC in addition to depressed contractile responses of the detrusor muscle by both cytotoxic drugs.

Mediator release from the urothelium/LP

In this study, a basal release of ATP, ACh and PGE₂ were observed in all tissues. Unlike the control tissues from the epirubicin and doxorubicin (aged) experiments, stretching the tissues resulted in increased ATP release over basal level in control bladders from the MMC experiments. This highlights the importance of running matched controls with each experiment as was done in this study so that changes caused by drug treatment can be clearly identified. Stretching the control tissues did not lead to an increase in ACh or PGE₂ in any of three experiments. Pretreatment with MMC and epirubicin has demonstrated various changes in these urothelial mediators.

The role of urothelial-derived ACh is still being identified but it has been suggested that once released in response to mechanical and chemical stimulation, urothelial-derived ACh may act in a paracrine manner to stimulate the detrusor muscle and sensory nerves, or in an autocrine manner to stimulate urothelial associated muscarinic receptors (Birder, 2010; Hanna-Mitchell et al., 2007). Kanai et al. (2007) applied muscarinic receptor agonist to rat bladder tissues and subsequently induced membrane-potential transients and Ca²⁺ transients that begin near the urothelial-lamina propria interface and then spread to the detrusor muscle. The study suggests the possibility that the urothelium may regulate the generation of spontaneous, non-voiding contraction in the bladder. Patients with OAB can be effectively treated with muscarinic receptor antagonists which enhance the storage phase of micturition by

inhibiting the binding of ACh at muscarinic receptors (M_2 & M_3) on the detrusor smooth muscle as well as pre-junctional muscarinic receptors in the bladder on cholinergic and adrenergic nerve terminals (Abrams and Andersson, 2007; Chess-Williams, 2002; Pathak and Aboseif, 2005; Staskin and MacDiarmid, 2006). Since, the full complement of muscarinic receptors are also expressed by the urothelium, this may indicate that urothelial-derived ACh can play a role in the OAB (Yoshida et al., 2006). In patients with OAB, increased cholinergic afferent effects, specifically sensitisation of ACh-evoked signalling in the urothelium have been reported (Yoshida et al., 2010). The results of this study revealed that in urothelial/LP tissues, basal and stretch-induced ACh release was elevated at the majority of time points by both cytotoxic drugs. However, this effect was only statistically significant at the 3 minute time point for stretch-induced ACh release after MMC and at the 7 minute time point for basal release after epirubicin. It appears that ACh release overall is increased by both cytotoxic drugs. Thus, enhanced release of ACh from the urothelium/LP following MMC and epirubicin pretreatment may contribute to overactivity of the bladder (increased urinary urgency and frequency) reported in patients following intravesical treatment with these two agents.

As it was discussed previously in doxorubicin study, it is well accepted that an augmented urothelial ATP release is associated with pathophysiology of bladder disorders such as OAB and IC/PBS. These conditions are characterised by increased bladder activity and painful sensations and it has been suggested that excitation of purinergic receptors on sensory fibers may be a contributing factor (Khera et al., 2004; Munoz et al., 2011; Smith et al., 2005; Sun et al., 2001). Therefore, depressed stretch-induced ATP release from the urothelium/LP following MMC and epirubicin treatment was surprising. Thus, urological adverse effects caused by intravesical MMC and epirubicin treatment such as dysuria, increased urinary urgency and frequency which are similar to those symptoms seen in OAB and PBS cannot be explained by reduced ATP release following MMC and epirubicin treatment.

However, there is some evidence suggesting that P2Y receptors might also be contributing to hyperexcitability and increased firing of bladder afferents that are associated with urinary urgency and pain, all of which are adverse effects experienced by patients treated with MMC and epirubicin. It has been reported that

P2Y₁, P2Y₂, P2Y₄ and P2Y₁₁ are expressed on urothelial cells (Birder et al., 2004; Chopra et al., 2008; Shabir et al., 2013) and respond to endogenous purine and pyrimidine nucleotides (ATP, ADP, AMP, adenosine, UTP and UDP) released into the extracellular environment from various tissues (Lazarowski and Boucher, 2001; Lazarowski and Harden, 1999; von Kugelgen, 2006). The function of these receptors in the bladder is not well studied, but there is a growing evidence to support the involvement of P2Y receptors in mechanosensation and or/ nociception under normal and pathological conditions. A recent study by Fry et al. (2012) has demonstrated enhanced spontaneous activity in rat bladder sheets after exogenous application of P2Y receptor agonist (ADP and UTP). Chen et al. (2010) has reported increased excitability of mouse bladder sensory neurons by UTP (a P2Y₂ and P2Y₄ agonist) via a depolarisation of the resting membrane potential, increasing action potential firing, and facilitating purinergic (P2X) currents. Also, a study by Timoteo et al. (2014) has shown that intravesical instillation of a P2Y₆ selective agonist in a rat cystometry model induced bladder overactivity characterised by increased urinary frequency, and also increased ATP release in the voided fluid. The urothelium expresses ecto-ATPase enzymes (Stella et al., 2010) and so is able to hydrolyse ATP to breakdown products such as ADP, AMP, and adenosine. The function of these enzymes is to limit the availability of ATP (Mansfield and Hughes, 2014e). It can be postulated that MMC and epirubicin treatment may increase the activity of ecto-ATPase activity in the urothelium, which leads to reduced ATP concentrations and increases the amount of ATP breakdown products. Thus, an increase in the hydrolysed products of ATP such as ADP, AMP and adenosine, which act on P2Y receptors, may be contributing to sensitisation of afferent pathway and increased bladder activity.

PGE₂, which is the product of the cyclooxygenase (COX) pathway, is also released from the urothelium during bladder filling. The actions of the urothelial-derived PGE₂ in the bladder are not well understood, but it has been reported to stimulate spontaneous bladder contractions (de Jongh et al., 2007), stimulate release of ATP from urothelial cells (Tanaka et al., 2011) and sensitize bladder capsaicin-sensitive afferent neurons (Maggi et al., 1988a), all actions which suggest PGE₂ may play important roles in the modulation of nerve and detrusor functions (Apodaca et al., 2007; Birder, 2005). Previous studies have shown that overexpression of PGE₂ in the bladder stimulates the micturition reflex through activation of C fibres (Aoki et al., 2009; Maggi et al., 1988a). Also, cyclooxygenase (COX), when inhibited improved

storage function in rats with detrusor overactivity, indicating potential stimulatory effect of PGE₂ on bladder micturition reflex (Yokoyama, 2010a). In humans, intravesical administration of PGE₂ caused detrusor overactivity, urgency and decreased bladder capacity (Schussler, 1990). In addition, elevated local PGE₂ production was reported in the bladders of animals with overactive detrusor muscle (Hu et al., 2003; Park et al., 1999) as well as patients with OAB (Kim et al., 2005; Kim et al., 2006a). Thus, enhanced basal PGE₂ release from the urothelium/LP following MMC pretreatment observed in this study may lead to increased activation of the bladder micturition reflex and may contribute to the increased urinary urgency and frequency experienced by patients treated intravesically with this drug. Unlike MMC treatment, epirubicin had no effect on the release of PGE₂.

The effects of urothelium-derived inhibitory factor (UDIF) on detrusor muscle contractility have been mentioned previously (Chapter 3). The influence of this factor was also evident in this study, where the presence of an intact urothelium/LP in control tissues significantly depressed maximum contractions of the detrusor muscle to carbachol. However, after MMC or epirubicin treatment inhibition of detrusor muscle responses by the urothelium/LP was absent. These findings suggest that MMC and epirubicin may depress the release or the actions of UDIF following urothelial damage which was represented by a decreased urothelial thickness as well as depressed contractile response of the urothelium/LP to carbachol by MMC. A reduction in inhibitory mechanisms of the urothelium on detrusor muscle contraction has been observed in the human neurogenic OAB. A study by Chess-Williams, Cross & Chapple (2009) demonstrated that inhibition of maximal responses to carbachol ($42.6 \pm 11.5\%$) in the presence of the urothelium in bladder strips from neurogenic OAB patients was less than that seen in control tissues ($65 \pm 4\%$). Thus, lack of inhibition on detrusor contractility by the urothelium/LP by the cytotoxic drugs observed in this study may also be a contributing factor to overactivity of the bladder.

Conclusions

In conclusion, it appears that MMC treatment is highly toxic on detrusor muscle as demonstrated in the present study by depressed contractile responses of the detrusor muscle to muscarinic receptor stimulation and neurogenic stimulation along with a reduced ability of detrusor muscle to relax to β -AR stimulation. Reduced detrusor

compliance may contribute to adverse effects such as decreased bladder capacity. Both the lack of UDIF effect on detrusor contractility and enhanced release of ACh and PGE₂ caused by toxicity of MMC on the urothelium (reduced urothelial thickness) would tend to result in bladder overactivity, thus causing increased urinary frequency and urgency. Epirubicin treatment, on the other hand, had a lower toxicity profile compared to MMC depressing contractile responses of detrusor muscle without affecting relaxation or neurogenic response. It appears to cause adverse effects through its toxic properties affecting mainly the urothelium (decreased urothelial thickness) causing abolition of the UDIF effect and increased ACh release both of which would tend to result in bladder overactivity.

Chapter 5 - The effects of mitomycin C on RT4 urothelial cells

Introduction

The previous chapters demonstrated that the cytotoxic agents doxorubicin, MMC and epirubicin alter porcine bladder function, in terms of muscle contraction, response to neurogenic stimulation and release of mediators from the urothelium/LP. A study conducted during my Honours project also demonstrated that doxorubicin alters release of urothelial mediators, ATP, ACh and PGE₂ from RT4 human urothelial cells, with stimulation of inflammatory cytokine release (Kang et al., 2013). Given that multiple cells types in the bladder wall have been reported to release mediators such as ATP, this chapter assessed the cell specific changes that occur when human urothelial cell cultures are treated with MMC. The work described in this chapter has recently been published in Naunyn-Schmiedeberg's Archives of Pharmacology in a paper entitled "Recovery of urothelial mediator release but prolonged elevations in interleukin-8 and NO secretion following mitomycin C treatment" by S. Kang, R. Chess-Williams, S. Anoopkumar-Dukie and C. McDermott.

Models of Urothelial Function

The urothelium which lines the luminal surface of the bladder is in close proximity to the cytotoxic agents during intravesical treatment. The urothelium was originally thought to act as a simple distensible barrier protecting the underlying nerves, muscular and vascular tissues from the potentially harmful contents of the urine. However, recent studies have shown that the urothelium can release a number of chemical mediators and transmitters including ATP (Burnstock, 2011), ACh (Birder, 2010; Hanna-Mitchell et al., 2007), PGE₂ (Aizawa et al., 2010; Apodaca et al., 2007; Birder, 2005; Tanaka et al., 2011) and NO (Birder et al., 1998; Giglio et al., 2005) in response to chemical and mechanical stimuli during bladder filling to communicate with underlying tissues, and regulate sensory mechanisms and bladder contraction (Birder et al., 2010f; Cockayne et al., 2000; Maggi et al., 1988a). Urothelial mediators have been covered in detail in Chapter 1.

Despite the urothelium acting as an effective barrier to the movement of substances from the bladder lumen to the deeper tissue layers, there is evidence that some intravesical chemotherapeutic agents diffuse into the detrusor muscle including MMC and doxorubicin, albeit only a fraction of the luminal concentration (Wientjes

et al., 1993; Wientjes et al., 1991). It is likely that the urothelium is the layer most affected by intravesical chemotherapy given the immediate contact between this layer and high concentrations of cytotoxic drugs. To determine the effects of MMC on urothelial function RT4 cells, a human urothelial cell line were used as a model of urothelial mediator release in this chapter.

Human cancer cell lines are widely used in research to examine cancer biology and develop anti-cancer drugs. Generally, it is only highly aggressive metastatic cancers that have accumulated the genetic changes necessary for unlimited growth *in vitro* that spontaneously become continuous cell lines. Therefore, most primary well-differentiated cancer cell lines are not mortal but still retain characteristics of normal urothelial cells (Masters, 2000). Thus, the RT4 cell line which is a malignant human urothelial cell line derived from a recurring papillary tumour of the bladder was used in this study, since it is from well-differentiated tumours, still retains features of asymmetric unit membrane and the specialised luminal surface of terminally differentiated superficial urothelial cells (Masters and Palsson, 1999). The *in vitro* urothelial culture model used in this study has been used previously to assess urothelial mediator release by our group and others (Kang et al., 2013; Mansfield and Hughes, 2014a, e; McDermott et al., 2012).

In vivo models are complicated by the presence of structural heterogeneity and do not allow for toxic mechanisms to be clearly defined. Using cultured urothelial cells allowed us to investigate the effects of MMC at cellular level and aids in identification of urothelial specific effects, in addition to performing repeat treatments and investigating the potential for recovery post-treatment. The complexity of bladder tissue is such that multiple cell types in the bladder wall release the mediators of interest in this study (Cheng et al., 2011). Given that our aim was to assess the effects of MMC on urothelial function, interpretation of changes in mediator release from intact tissue is complicated by multiple sources of release. A major limitation of animal studies is difficulty with interpretation based on species differences. Therefore, a significant advantage of the urothelial model used here is that it is of human origin and has been shown to release ATP, ACh and PGE₂ under basal and stretch conditions as in the intact bladder. We must however be mindful of the limitations of cell culture models when interpreting results and making conclusions that relate to the clinical setting due to possible changes to

functional characteristics in cultured cells and the complexity of the whole organism. Future pharmacological and physiological studies are therefore necessary to confirm if our conclusions regarding the role of inflammation are valid.

We recently reported that doxorubicin, at clinically relevant concentrations and durations of treatments, alters release of ATP, ACh and PGE₂ from RT4 urothelial cells (Kang et al., 2013). Twenty-four hours following treatment, urothelial mediator release was still affected and induction of pro-inflammatory cytokines (IL-8 and IL-1 β) was observed. Based on the results of this study, we hypothesised that release of inflammatory mediators may be a common factor following treatment with intravesical chemotherapeutic agents and many play a role in inducing local side effects.

Inflammation

Inflammation is triggered by tissue injury such as physical trauma, intense heat and irritating chemicals or when innate immune cells detect infection by viruses and bacteria (Khan and Khan, 2010; Newton and Dixit, 2012). Innate immune cells that reside in tissues including macrophages, fibroblasts, mast cells and dendritic cells, and circulating leukocytes such as monocytes and neutrophils recognise pathogen invasion or cell damage. Then, these immune cells as well as injured tissue cells release inflammatory mediators such as histamine, prostaglandins, and NO to cause vasodilation, which increases blood flow and brings in circulating leukocytes. Also, inflammatory mediators including histamine and leukotrienes act on endothelial cells to increase vascular permeability and allow plasma proteins and leukocytes to exit the circulation (Newton and Dixit, 2012). Cytokines, which is another inflammatory mediator, attract leukocytes to the site of inflammation and lastly, activated innate immune cells at the site of infection or injury, such as dendritic cells, macrophages and neutrophils, remove foreign particles by phagocytosis (Newton and Dixit, 2012).

Inflammation is reported in many bladder pathologies including IC/PBS and OAB. These disorders of the bladder have symptoms common to those reported by bladder cancer patients treated with intravesical chemotherapy such as cystitis including urinary urgency, together with frequency and dysuria (Macdiarmid and Sand, 2007; Thrasher and Crawford, 1992). There is much evidence demonstrating the

relationship between the inflammation of the bladder and the symptoms of urinary bladder diseases. Studies using cyclophosphamide (CYP)-induced cystitis model, which assessed cytokine expression, have detected enhanced IL-6, IL-1 α and IL-4, among others, protein and mRNA levels in the urine and urinary bladder (Eichel et al., 2001; Smaldone et al., 2009). Cystitis in rats has shown to increase bladder afferent excitability and is believed to contribute to bladder overactivity and pain (Hayashi et al., 2009). Increased transcription and expression of cytokine was observed in the urinary bladder of patients with ulcerative IC/PBS (Corcoran et al., 2013; Lotz et al., 1994; Ogawa et al., 2010). Also, several studies reported elevated IL-6 in the urine of patients with ulcerative IC/PBS (Erickson et al., 1997; Erickson et al., 2002; Lotz et al., 1994). Additionally, a recent study by Liu and her colleagues (2013) has demonstrated significantly increased serum cytokine (IL-1 β , IL-6, IL-8 and TNF- α) levels in OAB patients compared to the controls.

Cytokines are small proteins secreted by cells of the immune system that act in an autocrine, paracrine or endocrine manner to have a specific effect on the interactions and communications between cells to modulate inflammatory response. Cytokines include lymphokine (cytokines made by lymphocytes), monokine (cytokines made by monocytes), chemokine (cytokines with chemotactic activities), and interleukin (cytokines made by one leukocytes and acting on other leukocytes). Cytokines are redundant in their activity which means similar functions can be stimulated by different cytokines. Many cell types produce cytokines, but they are predominantly made by helper T cells and macrophages. Cytokines such as tumour necrosis factor (TNF) and interleukin-1 (IL-1) promotes recruitment of leukocytes at the site of inflammation through the process called “extravasation” by increasing the levels of leukocyte adhesion molecules on endothelial cells (Newton and Dixit, 2012; Sciacca et al., 1994). Interleukin-6 (IL-6) is also produced at the site of inflammation and plays a crucial role in inflammatory response. IL-6 is responsible for the transition from acute to chronic inflammation by altering the nature of leucocyte infiltrate (from polymorphonuclear neutrophils to monocyte/macrophages) and also favours chronic inflammatory responses by exerting stimulatory effects on T and B cells (Gabay, 2006). Interleukin-8 (IL-8) is also known for its role in inflammation and its main function is chemotaxis and activation of neutrophils and T cells in inflammatory region (Baggiolini, 1998). Other cytokines that plays a crucial role in inflammatory response include interleukin-10 (IL-10) and interleukin-12 (IL-12). IL-

IL-10 is a potent anti-inflammatory cytokine that limits host immune response to pathogens, thereby preventing damage to the host and maintaining normal tissue homeostasis (Iyer and Cheng, 2012). IL-12, which is produced mostly by phagocytic cells and to some degree by B-cells, induces cytokine production, primarily of interferon-gamma (IFN- γ), from natural killer (NK) and T cells, acts as a growth factor for activated NK and T cells, enhances the cytotoxic activity of NK cells, and favours cytotoxic T cell generation (Trinchieri, 1995). Hence, BD Cytometric Bead Array (CBA) Human Inflammatory Cytokine Kit, which can simultaneously quantify six cytokines (IL-8, IL-1 β , IL-6, IL-10, TNF and IL-12p70 are actively involved in inflammatory response as mentioned above), was used in this study to evaluate the effects of the three cytotoxic drugs on inflammation of the urothelium. Certain cytokines are involved in not only the initiation but also the persistence of pathologic pain by directly acting on nociceptive sensory neurons (Zhang and An, 2007). There are studies showing that inflammatory cytokines are released after intravesical treatment with immunotherapeutic agent, BCG (de Boer et al., 1997) and the chemotherapeutic agent, doxorubicin (Abou El Hassan et al., 2003). In addition, MMC has been shown to induce IL-8 release in human fibroblasts (Chou et al., 2007).

Nitric Oxide

NO, which is one of the non-adrenergic, non-cholinergic (NANC) transmitters affecting the bladder, is a small gaseous free radical with a half-life of less than 6 seconds (Andersson and Persson, 1994).

NO is an important cellular signalling molecule produced by nitric oxide synthase (NOS). There are three distinctive isoforms of NOS including endothelial nitric oxide synthase (eNOS), neuronal nitric oxide synthase (nNOS), and inducible nitric oxide synthase (iNOS) (Winder et al., 2014). It has been reported in several studies that NO formation occurs in urothelial cells via either iNOS or eNOS, with some reports also indicating the presence of nNOS mainly in species other than human (Birder et al., 2002; Chuang et al., 2013; Gillespie et al., 2005; Theobald, 2003).

NO activates soluble guanylate cyclase (sGC), a key enzyme of the NO-cyclic GMP (cGMP) signalling pathway which induces smooth muscle relaxation. Activation of

sGC results in formation of a second messenger, cGMP which activates protein kinase G. This causes reuptake of Ca^{2+} and opening of calcium-activated potassium channels. The decrease in intracellular concentration of Ca^{2+} activates myosin light-chain phosphatase (MLCP) which dephosphorylates the myosin light-chains, leading to smooth muscle relaxation (Bryan et al., 2009). The role of the NO-cGMP signalling pathway in regulating bladder smooth muscle (detrusor) relaxation is still unclear, however it appears to be the above mechanism (cGMP signalling pathway) which modulates high voltage Ca^{2+} channels, thereby affecting the firing threshold (Yoshimura et al., 2001).

NO is released from the urothelium in response to mechanical and chemical stimulation such as noradrenaline (NA), capsaicin and isoproterenol. It is a potentially important soluble factor which modulates bladder functions (Andersson and Persson, 1995; Apodaca et al., 2007; Birder et al., 2002; Birder et al., 2010g; Murakami et al., 2007). The role of NO in regulating bladder activity is still being evaluated but it may have several functions including relaxation of detrusor muscle, modulation of afferent and efferent nerve functions and regulation of urothelium barrier function (Birder et al., 2002).

The mechanical stimuli such as bladder filling will stimulate the release of NA which directly relaxes detrusor muscle. It also elicits release of NO which will enhance relaxation further. Also, stimulation of cholinergic receptors induces NO release from the urothelium which results in relaxation of the bladder outlet, allowing bladder emptying to occur with little increase in intravesical pressure (Apodaca et al., 2007; Chess-Williams, 2002). There is growing evidence which support the inhibitory effects of NO on the bladder reflex pathway. It has been reported that intravesical administration of oxyhemoglobin (NO scavenger) results in a bladder hyperactivity and also decreased bladder capacity inducing bladder contraction via modulation of the threshold for afferent nerve firing, indicating an inhibitory role for NO in the control of bladder reflexes (Pandita et al., 2000). In addition, an intravesically administered NO donor (*S*-nitroso-*N*-acetylpenicillamine) demonstrated decreased overactivity induced by chemical irritants in rats, further supporting the idea of NO partly exerting its effect on afferent nerve terminals (Ozawa et al., 1999). Furthermore, bladder overactivity of rat cystitis model induced by cyclophosphamide was further increased with an unselective NOS-inhibitor

(N(ω)-nitro-L-arginine methyl ester; L-NAME) (Andersson et al., 2011; Ozawa et al., 1999). These findings suggest that NO depresses activity of the bladder reflex by suppressing the excitability of and/or the release of transmitters from bladder afferent nerves (Masuda et al., 2007).

Contrarily, an excitatory effect of NO on bladder contractility has also been observed during electrical field stimulation (Liu and Lin-Shiau, 1997), possibly by aiding intracellular Ca^{2+} release in myocytes (Wei et al., 2008; Yanai et al., 2008). Also, some studies have discussed the role of NO in facilitating vesicular release of ACh and ATP which has been suggested to be responsible for the contractile effects of NO in the bladder (Bal-Price et al., 2002; Mothet et al., 1996).

In addition to the effects of NO on smooth muscle contractility in pathological conditions, studies in various tissues have suggested important immunomodulatory effects of NO on development of inflammation as well as necrosis/apoptosis and the subsequent loss of barrier function (Andersson et al., 2011; Chuang et al., 2013; Jezernik et al., 2003; Xu et al., 2002). Increased levels of NO in urine have been reported in patients with IC (Logadottir et al., 2004). The Ca^{2+} independent isoform, iNOS has been discussed by many studies as the key for the formation of urothelial NO in the acute phase of cell damage and early inflammatory response (Jezernik et al., 2003; Moncada et al., 1991; Persson et al., 1999). It has been shown that lipopolysaccharide (LPS) treatment can cause upregulation of iNOS in the urothelium (Persson et al., 1999). Hence, it can be postulated that subsequent apoptosis and disrupted barrier function is due to high levels of NO produced as a result of iNOS (Jezernik et al., 2003). This is further supported, since a decrease in urothelial damage and subsequent inflammation have been observed by preventing the formation of NO with a NOS-inhibitor (Souza-Fiho et al., 1997). Furthermore, the expression of another isoform, eNOS is increased in the urothelium and suburothelium of rats 60 hours following treatment with the anti-cancer agent cyclophosphamide when the inflammatory response was at its peak (Giglio et al., 2005).

Aims

Generally, intravesical treatments are received at weekly intervals. To our knowledge, recovery of urothelial function over longer periods such as this has not been assessed to date. The aim of this study was to investigate the effects of MMC on basal and stretch-induced release of urothelial mediators (ATP, ACh and PGE₂) from human urothelial cells and determine whether changes in urothelial function may provide an explanation for the urological adverse effects experienced by patients treated with intravesical MMC. The release of inflammatory cytokines and NO from urothelial cells after MMC treatment was also assessed to determine if inflammation may play a role in the adverse effects associated with treatment. In addition, recovery of urothelial function was assessed 1 week post-treatment and repeat treatments conducted.

Materials and Methods

Cell culture

The RT4 cell line is an immortalised human urothelial cell line derived from explants of a recurring papillary tumour of the bladder supplied by European Collection of Cell Cultures (ECACC). RT4 cells were routinely cultured in 75cm² culture flasks (T75) using McCoy's 5A culture medium supplemented with additional 2mM L-glutamine, 1% v/v penicillin-streptomycin and 10% v/v Fetal Bovine Serum (FBS). Cells were maintained at 37°C in 5% CO₂. The doubling time of RT4 cells is approximately 36 hours and confluent cells (approx. 90%) were subcultured at a 1:4 ratio by detaching cells from the flask using a trypsin/EDTA solution.

Seeding densities of cell treatments

Cell viability was assessed using trypan blue exclusion prior to seeding plates for treatment with mitomycin C (MMC). Only cells with viability $\geq 95\%$ were used for experiments. RT4 cells were seeded in 24 or 96 well plates or T25 flasks at a density determined in initial optimisation experiments, see Table 5.1 below. Cells were incubated overnight at 37°C to allow cells to adhere. Seeding density was also dependent on the timing of endpoint measurement following initial or repeat treatment.

Table 5.1: Optimum seeding density used for experiments with RT4

Timing of Endpoint Measurement	Seeding Density in 96 well plates	Seeding Density in 24 well plates	Seeding Density in T25 flasks
Immediate	0.05×10^6 per well	0.3×10^6 per well	4.0×10^6 per well
24 hours after 1 st treatment	0.015×10^6 per well	0.1×10^6 per well	1.3×10^6 per well
1 week after 1 st treatment	0.085×10^5 per well	0.05×10^6 per well	0.65×10^6 per well
1 week after 2 nd treatment	0.05×10^5 per well	0.03×10^6 per well	0.4×10^6 per well

MMC treatment of urothelial cells

Krebs-bicarbonate solution was added to the first column of the plate serving as a vehicle control (untreated) (Figure 5.1). The next 5 columns were treated with increasing concentrations of MMC (0.0002, 0.002, 0.02, 0.2 and 2mg/ml). MMC was diluted in Krebs-bicarbonate solution and was allowed to equilibrate for 30 minutes at 37°C immediately before use. The plate was then incubated for two hours at 37°C in 5% CO₂. Cell viability and urothelial mediator release was then assessed immediately post-treatment.

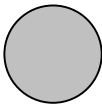
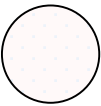
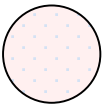
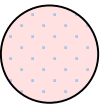
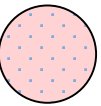
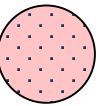
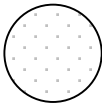
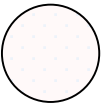
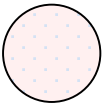
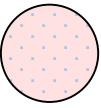
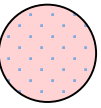
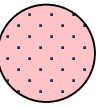
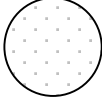
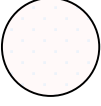
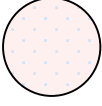
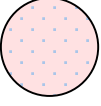
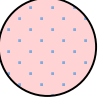
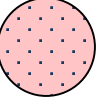
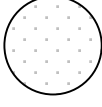
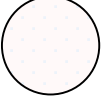
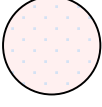
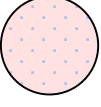
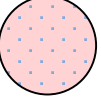
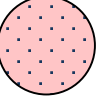
Control (Krebs)	MMC (0.0002mg/ml)	MMC (0.002mg/ml)	MMC (0.02mg/ml)	MMC (0.2mg/ml)	MMC (2mg/ml)
					
					
					
					

Figure 5.1: Illustration of 24 well plate containing control and MMC treated wells.

To assess the late effects (24 hour post 1st treatment) of MMC on urothelial function, cell viability, NO and cytokine release, after treatment with MMC concentration of 0.0002 or 0.02 mg/ml for 2 hour, all solutions on the plate were aspirated, cells were washed twice with PBS. McCoy's 5A culture medium was added into each well seeded. The plate was then incubated at 37°C for a further 24 hours prior to analysis of endpoints.

To assess effects of MMC on treated cells 1 week after 1st treatment with 0.002 mg/ml MMC, plates/flask were handled as just described but incubated at 37°C for 1 week. The culture medium was changed every two days during this period.

Urothelial cells were treated with lower concentrations of MMC for 24 hour (0.0002 or 0.02 mg/ml) and 1 week (0.002 mg/ml) post-treatment experiments as a previous

study showed that the concentration of MMC that reaches the urothelial cells is approximately 3% of intravesical treatment (Wientjes et al., 1993). They also report that the amount of MMC penetrating the normal bladder tissue is lower than the levels penetrating the superficial tumours of the bladder. In addition, often the dose used clinically is half of that used here (Colombo et al., 2012). Therefore, these lower concentrations are more likely to reflect the concentration reached in urothelial cells *in vivo*. In addition, higher concentrations of MMC left cell viability too low to allow for measurement of urothelial mediator release as would be expected with such a high concentration. Since cultured urothelial cells are actively proliferating unlike urothelial cells *in situ*, they are likely to be more vulnerable to the cell death mechanisms of chemotherapeutic agents such as MMC that tend to be more specific for proliferating cells. Hence, it is more appropriate to assess the drug at concentrations that have little effect on cell viability.

Repeat MMC treatment of urothelial cells

To assess the effects of repeated treatment on urothelial cells, cells received a 2nd treatment with the same concentration of MMC 1 week following initial treatment. Cells were initially handled in the same way as those just described for analysis of endpoints following 1 week with culture medium being changed every two days. After 1 week incubation, treatment wells were again exposed to 0.002 mg/ml of MMC (2nd treatment) for two hours, while control wells were exposed to normal Krebs-bicarbonate solution. After second treatment, incubation medium was aspirated and cells were washed with PBS. Fresh McCoy's 5A culture medium was then added into each well. The plate was then incubated at 37°C for further 1 week. The culture medium was again changed every two days. After 1 week incubation, endpoint analysis was carried out as described in the following sections.

Assessment of urothelial cell viability - resazurin reduction assay

Cells were seeded in 96-well microtiter plates as previously described and treated with MMC (0-2mg/ml) for 2 hour at 37°C. Immediately, 24 hour and 1 week after 1st MMC treatment, and also 1 week after 2nd MMC treatment, fresh medium containing 44 µM resazurin was added to each well. After 2 hour incubation at 37°C, the quantity of resorufin was determined by fluorescence (excitation 530 nm; emission 590 nm) using a Modulus microplate reader. Appropriate cell free controls were also included. Under all conditions tested, the extent of resazurin reduction was directly proportional to viable cell counts (data not shown). No change in cell number was observed immediately after 2 hour MMC treatment. Viability was also assessed 7 days after repeat MMC treatment.

Mediator release from treated cells

Basal release

Immediately, 24-hour, or 1-week following 2 hour incubation with MMC, all solutions on the plate were aspirated and cells were washed twice with phosphate buffered saline (PBS) solution.

Preparation of samples for analysis of mediator release from RT4 cells was previously described by Kang et al (2013). Krebs-bicarbonate solution (150µl) was added into each well (Figure 5.2). The plate was then incubated at 37°C. After 15 minutes, the solutions in each well were collected into microfuge tubes and these are representative of basal release of urothelial mediators from MMC treated and control cells.

Hypo-osmotically stimulated release

Hypotonic Krebs-bicarbonate solution was added into each well (150µl) (Figure 5.3). The use of hypotonic Krebs-bicarbonate solution (50% of normal NaCl content; 180 mOsm/L) causes the cells to swell, mimicking stretching of the cells (Hanna-Mitchell et al., 2007; Kang et al., 2013). After 15 minutes incubation at 37°C, the solutions in each well were collected into microfuge tubes.

Samples collected from each row were used for measuring each of the urothelial mediators (ACh, ATP and PGE₂) (Figure 5.2 and 5.3). This collection of samples

containing urothelial mediators from basal and stimulated conditions equals one independent experiment (N=1) and samples from each experiment were frozen at -80°C for later assay of urothelial mediators.

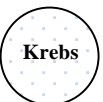
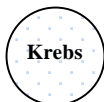

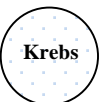
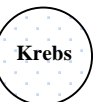
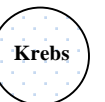


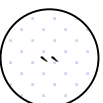




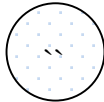


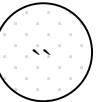
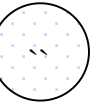
	Control (Krebs)	MMC (0.0002mg/ml)	MMC (0.002mg/ml)	MMC (0.02mg/ml)	MMC (0.2mg/ml)	MMC (2mg/ml)
Samples for ACh assay						
Samples for ATP assay						
Samples for PGE₂ assay						

Figure 5.2: Illustration of control and mitomycin C treated plate exposed to Krebs-bicarbonate solution (or isotonic solution) for measurement of basal mediator release.












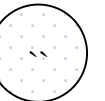




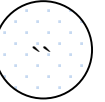
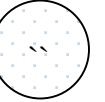
	Control (Krebs)	MMC (0.0002mg/ml)	MMC (0.002mg/ml)	MMC (0.02mg/ml)	MMC (0.2mg/ml)	MMC (2mg/ml)
Samples for ACh assay						
Samples for ATP assay						
Samples for PGE₂ assay						

Figure 5.3: Illustration of control and mitomycin C treated plate exposed to hypotonic Krebs-bicarbonate solution (or hypotonic solution) for measurement of hypo-osmotically stimulated mediator release.

Inflammatory cytokine and NO release from treated cells

To assess the late effects (24 hour post 1st treatment) of MMC on release of inflammatory cytokines and urothelial NO, cells were seeded in 25cm² flasks at density and treated with MMC concentrations (and duration), as described above. Control flasks were exposed to normal Krebs-bicarbonate solution (untreated). The flasks were then incubated for 2 hour at 37°C in 5% CO₂.

After treatment, solutions on each flask were washed off. McCoy's 5A culture medium was added into each flask. The flasks were then incubated at 37°C for 24 hours.

After 24 hour incubation, samples of cell free incubation medium for analysis of cytokines (IL-8, IL-1 β , IL-6, IL-10, TNF and IL-12p70) and NO metabolites (nitrate and nitrite) were collected. The samples were frozen at -80°C until analysis. In additional experiments, samples were also collected 1 week following 1st and 2nd treatment, with media changes performed every 2 days during the incubation period.

(The release of various mediators was measured using commercially available kits as described in “Assays for urothelial mediators, NO and inflammatory cytokines” section in Chapter 2)

Results

Immediate effects of MMC on urothelial mediator release

Immediately following treatment, no significant change was observed in basal release of ATP from human urothelial cells at all concentrations of MMC tested (Figure 5.4a). Stimulated release of ATP was observed in vehicle treated control urothelial cells and cells treated with MMC (0.0002-0.2mg/ml), with significant increase above basal levels of ATP release in response to hypotonic stimulation; except at 2mg/ml where stimulated release of ATP was completely abolished (95% decrease) (Figure 5.4b).

Immediately following MMC treatment, whilst basal release of ACh from urothelial cells exposed to 2mg/ml was significantly increased (2-fold) compared to vehicle treated control cells; stimulated release of ACh was not significantly affected by MMC in comparison to control (Figure 5.4c and d).

Basal release of PGE₂ from cells treated with 2mg/ml of MMC was significantly decreased (7-fold) immediately following 2 hour drug exposure compared to control (Figure 5.4e). A significant decrease in release of PGE₂ in response to hypotonic stimulation was observed in cells treated with MMC (0.0002-0.2mg/ml) (Figure 5.4f). In contrast, there was a significant increase in stimulated release of PGE₂ from cells treated with 2mg/ml of MMC.

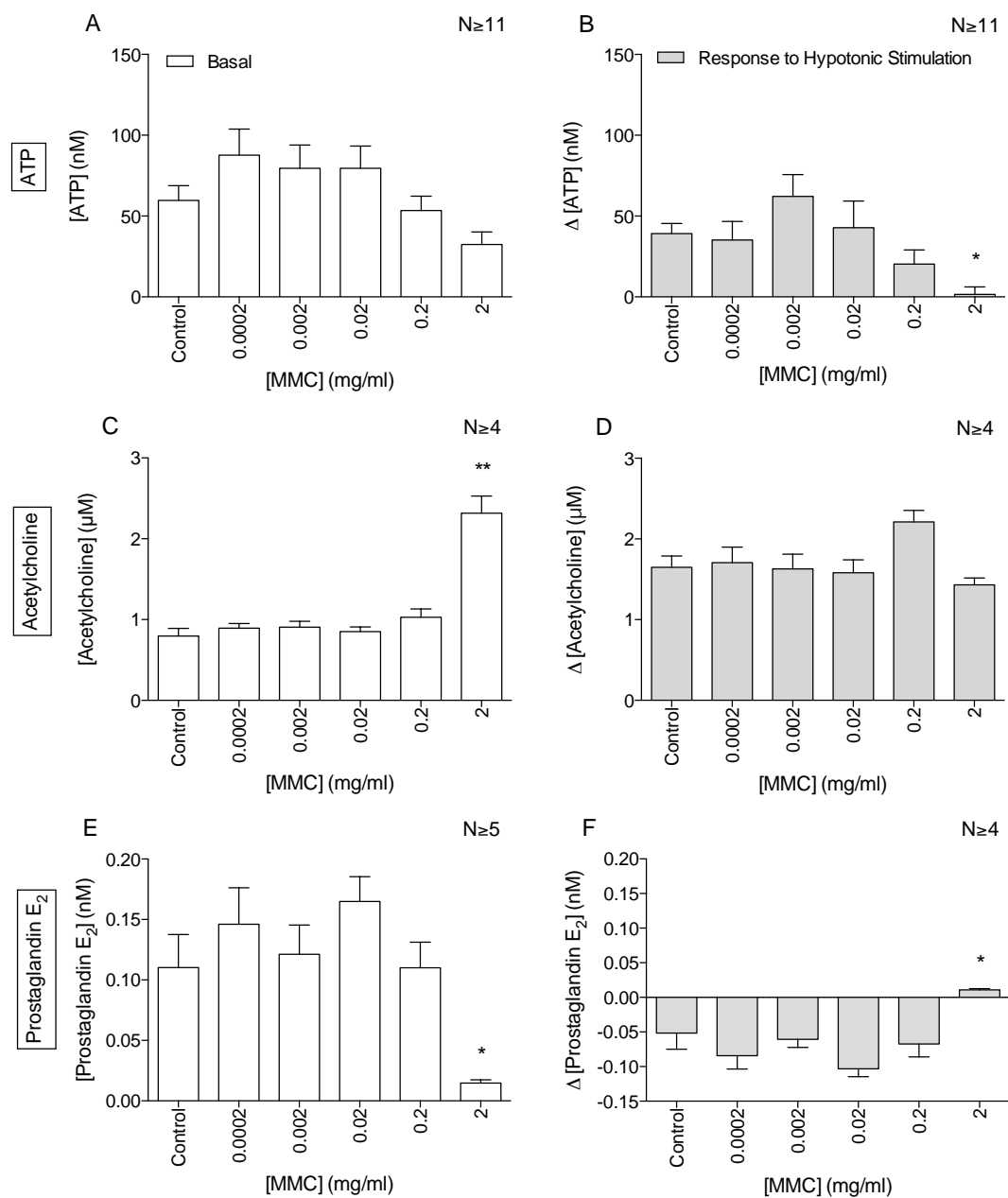


Figure 5.4: Immediate effects of 2 hour mitomycin C (MMC) treatment on basal extracellular ATP [A], acetylcholine [C] and prostaglandin E₂ [E] concentrations, and ATP [B], acetylcholine [D] and prostaglandin E₂ [F] response to hypo-osmotic stimulation. Data are expressed as means \pm SEM. *P<0.05 and **P<0.01 compared to vehicle control.

Effects of MMC on urothelial cell viability and mediator release

24h post-treatment

Twenty-four hours following MMC pre-treatment a concentration-dependent decrease in urothelial cell viability was observed, with significant changes detected in cells treated with MMC ≥ 0.0002 mg/ml (Figure 5.5a).

Since urothelial mediator release is affected immediately after MMC treatment, the ability of the urothelium to recover was assessed. Release of urothelial mediators was measured 24 hour after MMC pre-treatment with lower MMC concentrations, as these are more likely to reflect the concentration reached in urothelial cells *in vivo*, which has been estimated to be 3% of intravesical concentration (Wientjes et al., 1993). Both basal and stimulated release of ATP from MMC pretreated urothelial cells remained unchanged compared to vehicle-treated controls 24 hour following treatment (Figure 5.6a and b). In contrast, 24 hour after MMC treatment, concentration-dependent increase in basal ACh release was observed with significant increases at both 0.0002 and 0.02mg/ml (Figure 5.6c) but stimulated ACh remained unchanged compared to controls (Figure 5.6d). In addition, basal release of PGE₂ was significantly increased in urothelial cells pre-treated with 0.02mg/ml MMC compared to control cells (Figure 5.6e) and PGE₂ response to stretch was not observed in both controls and MMC pretreated cells 24 hour following treatment with basal release being significantly higher than stimulated release at 0.02mg/ml MMC (Figure 5.6f).

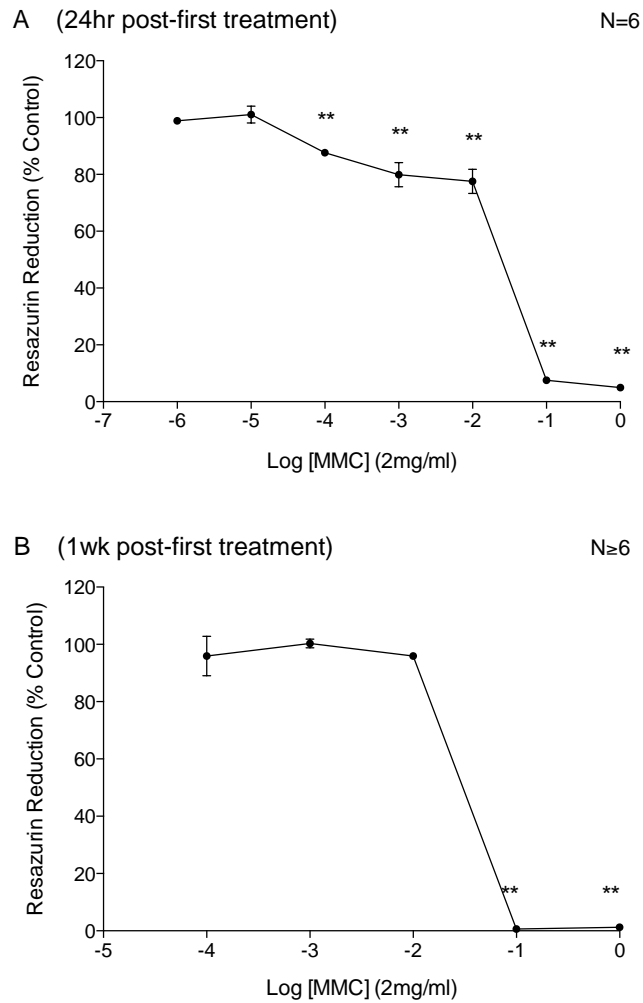


Figure 5.5: Effect of 2 hour mitomycin C (MMC) treatment on urothelial cell viability assessed by resazurin reduction 24 hour [A] and 1 week [B] after first treatment. Data are expressed as means \pm SEM. **P<0.01 compared to vehicle treated control cultures.

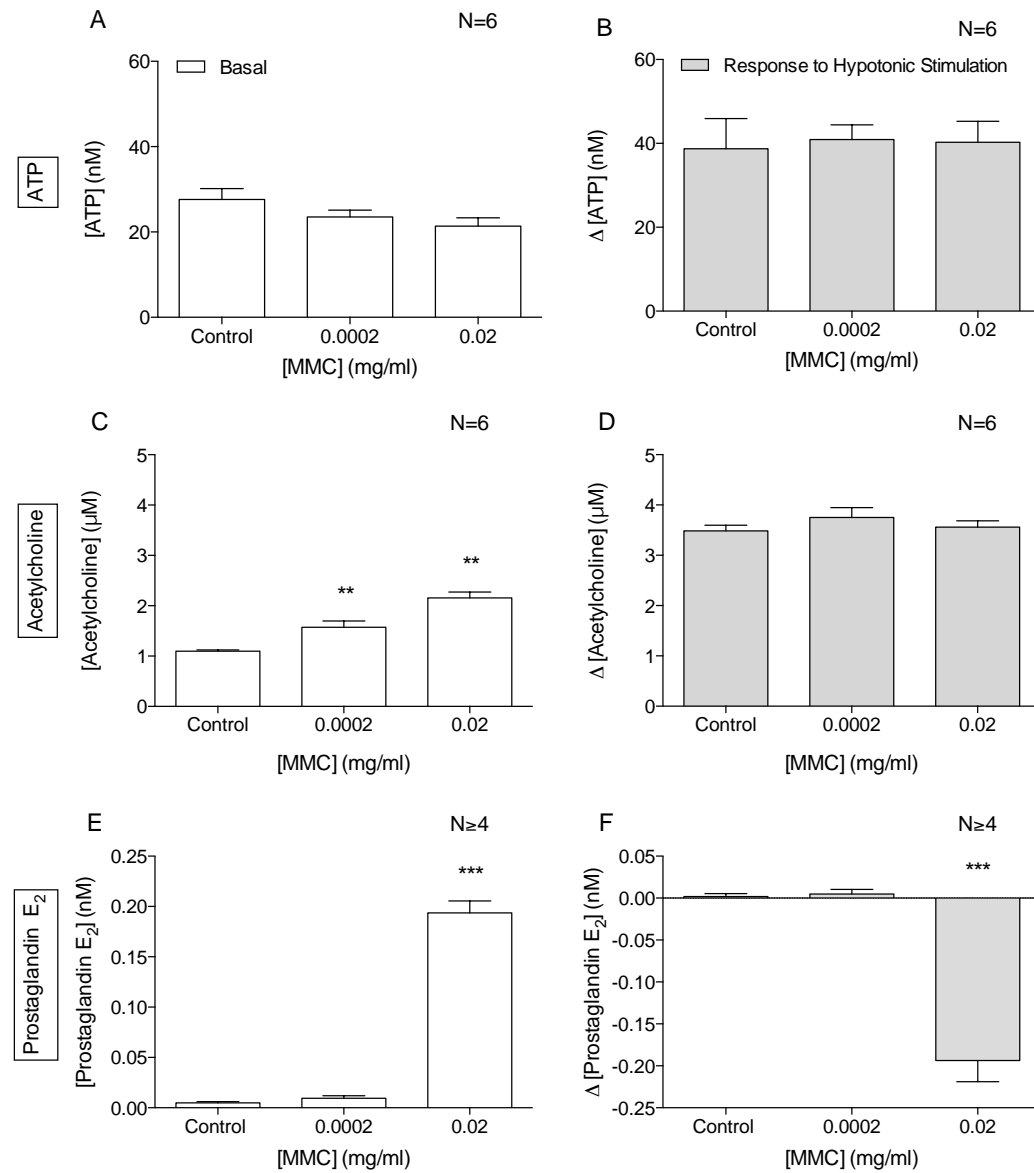


Figure 5.6: Twenty-four hour post-treatment effects of mitomycin C (MMC) on basal extracellular ATP [A], acetylcholine [C], prostaglandin E_2 [E] concentrations, and ATP [B], acetylcholine [D] and prostaglandin E_2 [F] response to hypo-osmotic stimulation. Data are expressed as means \pm SEM. ** $P < 0.01$ and *** $P < 0.001$ compared to vehicle control.

Effects of first and repeat MMC treatments on urothelial cell viability and mediator release at 1 week

One week following MMC pre-treatment urothelial cell viability was not affected in cells treated with $\text{MMC} \leq 0.02\text{mg/ml}$, however complete cell death were observed in cells treated with 0.2 and 2mg/ml MMC (Figure 5.5b). One week after MMC pre-treatment with 0.002mg/ml MMC concentration, stimulated release of ATP was significantly decreased in pre-treated cells compared to control cells (Figure 5.7a and b). While basal release of ACh was significantly decreased, stimulated release of ACh was significantly increased in pre-treated cells (Figure 5.7c and d). Also, in MMC pre-treated cells, basal release of PGE_2 was significantly increased compared to control cells (Figure 5.7e and f).

To assess the long-term (1 week) and repeat treatment effects of MMC, urothelial mediator release from cells treated with 0.002mg/ml MMC was assessed. This treatment concentration was selected to ensure adequate viable cell numbers for endpoint experiments. Urothelial cell viability was reduced to $86.64 \pm 5.7\%$ 1 week after repeat MMC treatment with 0.002mg/ml. No significant change in ATP (Figure 5.8a and b) and ACh release (Figure 5.8c and d) was observed one-week after repeat MMC treatment (0.002mg/ml), but stimulated release of PGE_2 was significantly increased in cells pre-treated with 0.002mg/ml MMC compared to control cells (Figure 5.8e and f). Response of all three mediators to stretch was not observed in control cells.

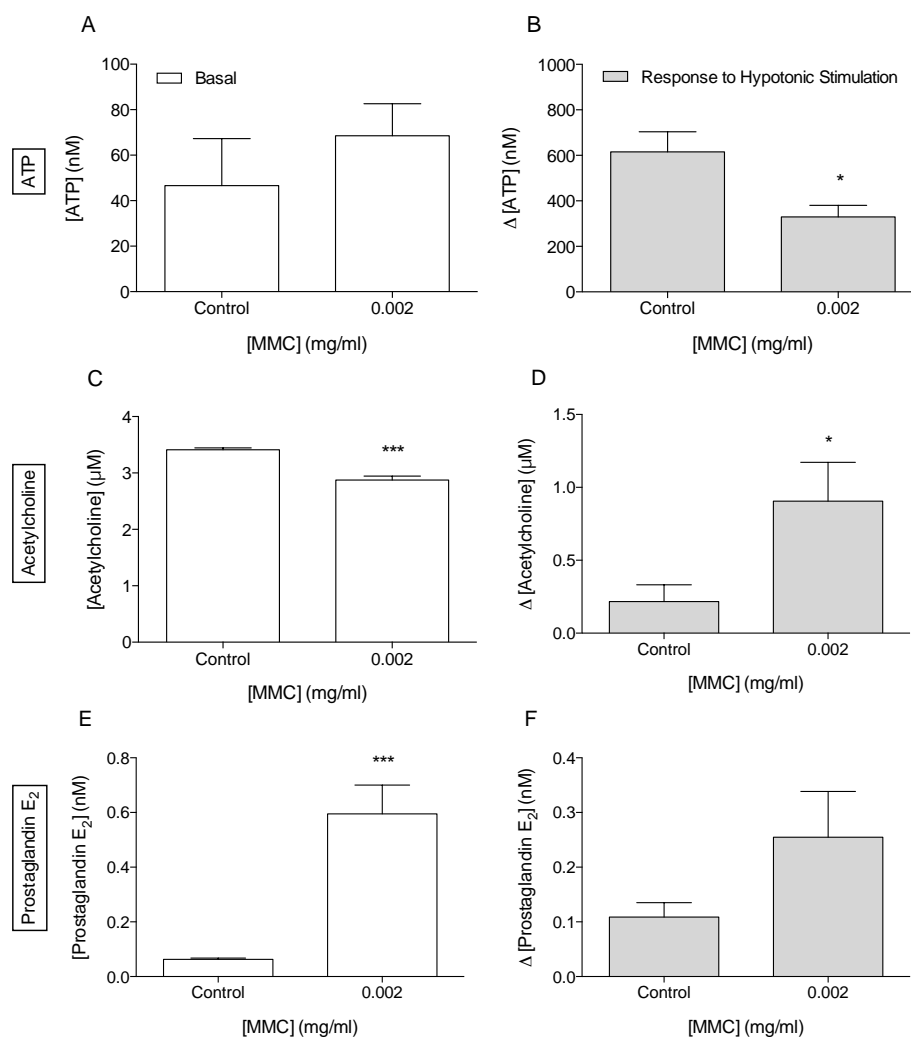


Figure 5.7: One week post-first treatment effects of mitomycin C (MMC) on basal extracellular [A] ATP, [C] acetylcholine, [E] prostaglandin E₂ concentrations, and [B] ATP, [D] acetylcholine and [F] prostaglandin E₂ response to hypo-osmotic stimulation. Data are expressed as means ± SEM (N=6). *P<0.05, **P<0.01 and ***P<0.001 compared to vehicle control.

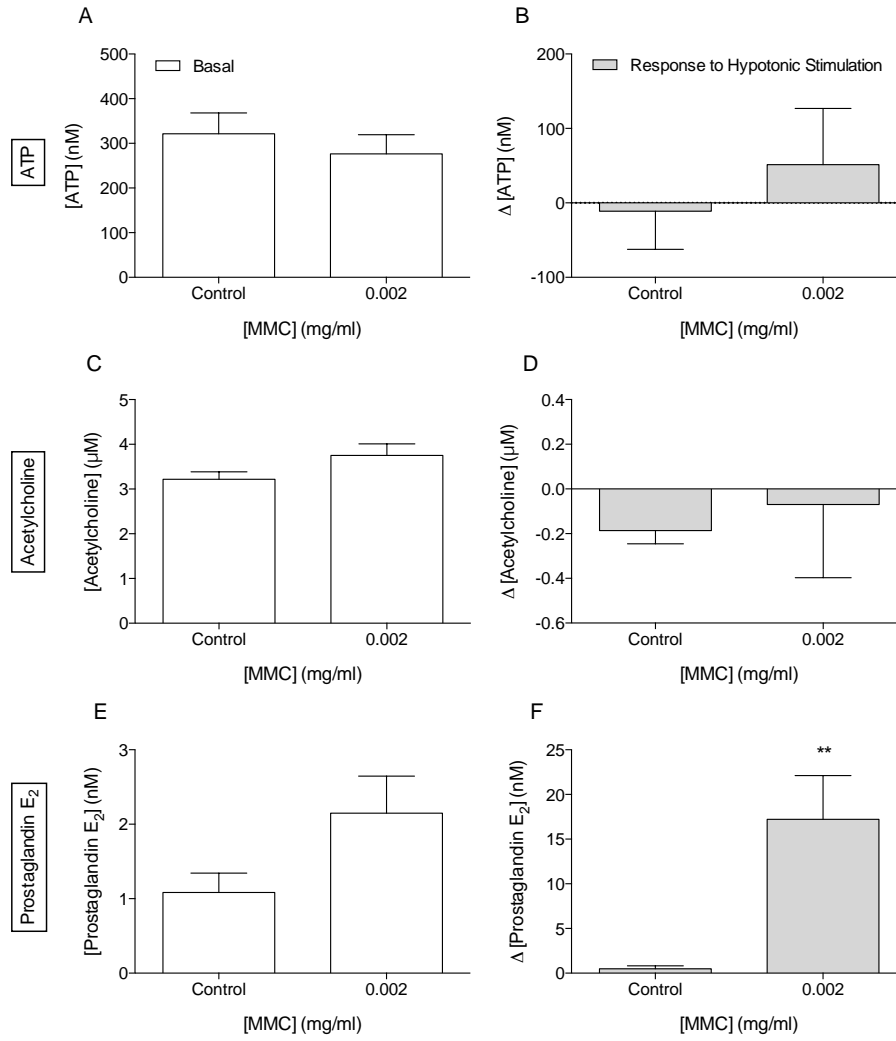


Figure 5.8: One week post-repeat treatment effects of mitomycin C (MMC) on basal extracellular [A] ATP, [C] acetylcholine, [E] prostaglandin E₂ concentrations, and [B] ATP, [D] acetylcholine and [F] prostaglandin E₂ response to hypo-osmotic stimulation. Data are expressed as means \pm SEM (N=6). *P<0.05 and **P<0.01 compared to vehicle control.

Effects of MMC on inflammatory cytokine and NO release

The presence of inflammatory cytokines (Interleukin (IL)-8, -1 β , -6, -10, -12p70 and tumour necrosis factor) in urothelial cell incubation medium was tested 24 hour and 1 week after 1st MMC treatment and also 1 week after 2nd MMC treatment as a measure of urothelial inflammation. IL-8 was secreted by untreated urothelial cells at 24 hour, while urothelial cells treated with MMC (0.02mg/ml) showed a 326-fold increase in release (25.52 ± 2.53 pg/ml control vs. 8321 ± 654 pg/ml treated, $P < 0.01$) (Figure 5.9a). IL-1 β , -6, -10, -12p70 and tumour necrosis factor were not detected in control or treated samples at any timepoint tested. Increased IL-8 secretion was also detected 1 week after 1st (13-fold increase from) and repeat (7-fold increase compared to) MMC treatment (Figure 5.9b and c).

Metabolites of NO (nitrate and nitrite) were measured in incubation medium to determine the effects of MMC on urothelial NO release. NO was released by untreated urothelial cells at 24 hour, with a significant increase observed from cells treated with MMC (0.02mg/mL) (Figure 5.10a). A significant increase in urothelial release of NO was also evident 1 week after the 1st MMC treatment (Figure 5.10b), but 1 week after 2nd MMC treatment no significant differences could be detected compared to control (Figure 5.10c).

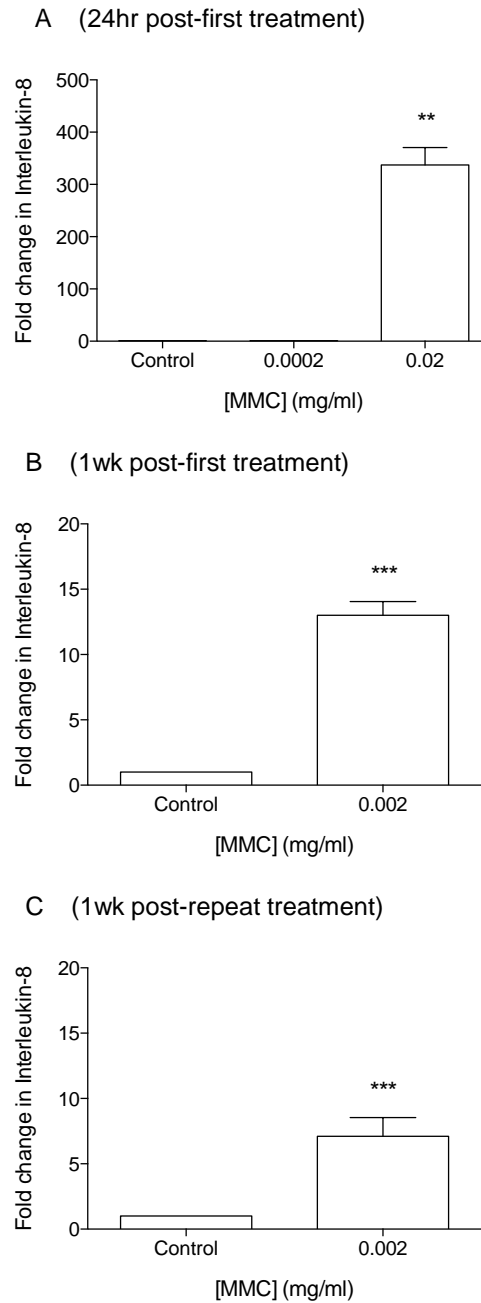


Figure 5.9: Interleukin-8 secreted from control and mitomycin C (MMC) treated urothelial cells [A] 24 hour, [B] 1 week after first treatment and [C] 1 week after repeat treatment. Data are expressed as means \pm SEM (N=6). **P<0.01 and ***P<0.001 compared to vehicle control.

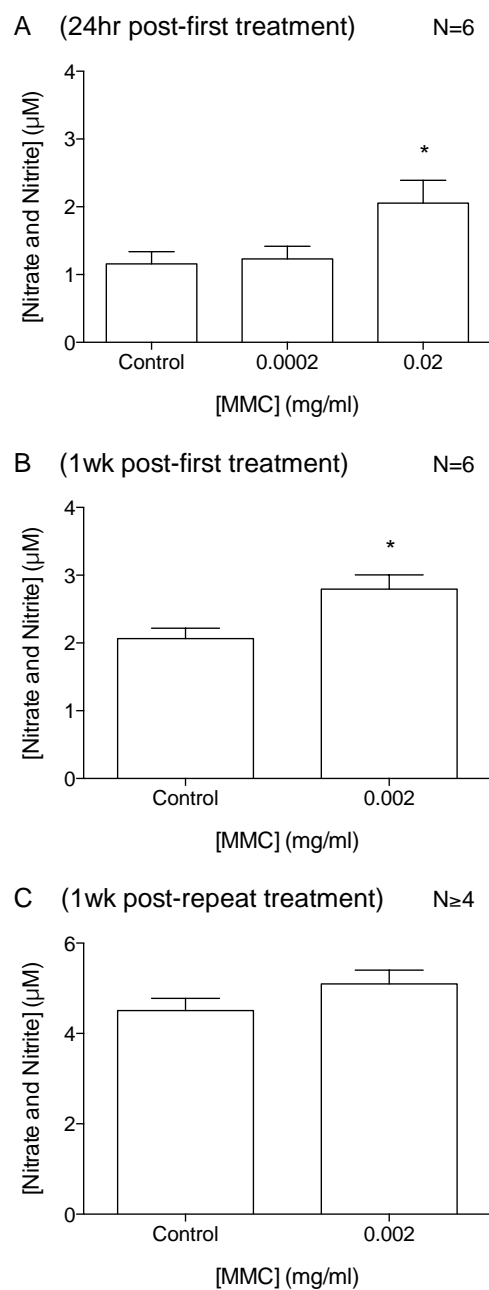


Figure 5.10: Effect of mitomycin C (MMC) on metabolites of nitric oxide (nitrate and nitrite) 24 hour [A], 1 week [B] after first treatment and 1 week [C] after repeat treatment. Data are expressed as means \pm SEM. * $P < 0.05$ compared to vehicle control.

Discussion

We previously reported that doxorubicin treatment results in alterations in the release of urothelial mediators (ATP, ACh and PGE₂) immediately and 24 hour following treatment (Kang et al. 2013). In addition, there are strong links between development of bladder symptoms and inflammation in other conditions (Nomiya et al., 2012; Yoshimura et al., 2002) and the cytotoxic agent doxorubicin has been shown to increase urothelial secretion of interleukin -8 and -1 β (Kang et al., 2013). Thus, we investigated the effects of the commonly used intravesical chemotherapeutic agent MMC on urothelial function, markers of inflammation and the ability to recover following first and repeat treatments.

ATP

As discussed previously in Chapter 3, an increase in ATP release is closely associated with disorders of the bladder such as OAB and IC/PBS that share symptoms similar to those local adverse effects experienced by patients treated with intravesical MMC. Therefore, in this study, enhanced release of ATP from the urothelial cells was expected by MMC treatment. Surprisingly, MMC had an inhibitory effect on stimulated release of ATP at the clinical concentration (2mg/ml) immediately after treatment. Twenty-four hours following MMC treatment, stimulated release of ATP was recovered back to control level but after 1 week recovery period, MMC had again inhibitory effect on stimulated release of ATP at MMC concentration 1000-fold lower (0.002mg/ml) than the clinical concentration. However, repeat MMC treatment had no effect on stimulated ATP release following another 1 week recovery period, suggesting that ATP release is unlikely to play a major role in the adverse effects induced by MMC treatment in the long-term. Similarly, doxorubicin has inhibitory effects on urothelial ATP release (Kang et al., 2013). The urological side effects experienced by patients following intravesical MMC treatment cannot be explained by inhibition in stimulated ATP release reported here. The discrepancy may be addressed by various factors. One is that the pain induced by MMC treatment may be due to increased sensitivity of afferent C fibers rather than the increase in ATP release that is observed in other bladder disorders mentioned previously. Decreased ATP release reported here may not

directly take part in causing urinary symptoms but may impact on the release of other urothelial mediators, potentially leading to abnormal communication between the urothelium and underlying layers such as myofibroblasts, nerves and detrusor muscle. Another possibility is that there may be enhanced release of ATP from other cells in the bladder wall resulting in symptoms of pain. Both interstitial cells and smooth muscle cells from the bladder have been shown to release ATP during stretch (Cheng et al., 2011). Furthermore, the decrease in urothelial ATP release may be due to MMC acting on the ecto-ATPase enzyme to enhance ATP breakdown, which in turn the hydrolysed products of ATP and itself can act on P2Y purinergic receptors present on the urothelium (Lazarowski and Boucher, 2001; Lazarowski and Harden, 1999; von Kugelgen, 2006). As discussed in Chapter 4, activation of P2Y purinoceptors is associated with overactivity and increase firing of bladder afferents that can cause urinary urgency and pain (Chen et al., 2010; Fry et al., 2012; Timoteo et al., 2014).

Acetylcholine

Unlike, unaltered ACh release from MMC pretreated porcine urothelial/LP strips discussed in Chapter 4, MMC treatment affected release of ACh from human urothelial cells. The potential excitatory role of urothelial-derived ACh in bladder micturition reflex as well as its association with bladder pathologies such as OAB was reviewed in detail from the previous chapter (4). Thus, increased release of basal ACh from the urothelial cells immediately and 24 hour following MMC treatment as well as increased release of stimulated ACh release one week following MMC treatment may activate cholinergic transmission in the bladder, possibly leading to the bladder overactivity observed in patients treated with intravesical MMC. The implication of inhibited release of basal ACh one week following MMC treatment in causing overactivity of the bladder is unknown. However, it may be postulated that a decrease in ACh release from the urothelium could cause an imbalance in cholinergic systems in the bladder, possibly leading to urinary dysfunction. However, since repeat MMC treatment had no effect on ACh release after a recovery period of one week, it is unlikely that ACh release like ATP is involved in adverse effects of MMC treatment in the long-term.

Prostaglandin E₂

In this study, MMC had an inhibitory effect on basal release of PGE₂ immediately following treatment with the clinical dose. This may be due to changes in ATP release that were observed immediately after treatment as these two mediators are reported to be co-related (Tanaka et al., 2011). In contrast, 24 hour following treatment with a 100-fold lower MMC concentration, an excitatory effect of MMC on basal release of PGE₂ was observed. Also, one week following treatment with a 1000-fold lower MMC concentration, MMC had an excitatory effect again on basal release of PGE₂. Doxorubicin has also been shown to increase basal but also stimulated release of PGE₂ from urothelial cells (Kang et al., 2013). In addition, stimulated release of PGE₂ was enhanced one week following repeat treatment. Since there is growing evidence indicating potential stimulatory effects of PGE₂ on bladder micturition reflex and its association with bladder pathology such as OAB as mentioned in Chapter 4, these findings suggest that unlike ATP and ACh, PGE₂ release is affected long-term and that sustained enhancement of PGE₂ release by MMC may lead to a sensitisation of the micturition reflex, possibly causing increased urinary frequency as well as perception of pain which are side effects commonly experienced by patients treated with MMC. Therefore, while less ATP release was observed here following MMC treatment, sensitisation of nerves due to the enhanced PGE₂ release reported would mean that less ATP is required to stimulate sensory activity.

Inflammatory cytokine

Release of inflammatory mediators is associated with intravesical Bacillus Calmette-Guerin (BCG) treatment (de Boer et al., 1997) and has also been linked to chemotherapeutic agents such as doxorubicin (Abou El Hassan et al., 2003). Previous studies have also shown that MMC induces release of IL-8 in human fibroblasts (Chou et al., 2007). We have also recently reported that the chemotherapeutic agent doxorubicin induces release of IL-8 and IL-1 β from treated urothelial cells (Kang et al., 2013). Here we assessed the effects on MMC on release of a number of human inflammatory cytokines from urothelial cells but only IL-8 was detected, with a 326-fold increase 24 hours after treatment. IL-8 was still elevated 1-week after treatment and again following repeat MMC treatment, while

release of urothelial ATP and ACh had recovered after repeat treatment. It was reported by Mansfield and Hughes (2014a) that exposure of human urothelial cells to inflammatory mediators (histamine and serotonin) resulted in decreased release of stretch-induced ATP, suggesting that decreased ATP release observed here is possibly due to the effects of elevated IL-8 (inflammatory cytokine) release by MMC treatment. Inflammation has been shown to increase bladder afferent excitability and is believed to contribute to bladder overactivity and pain (Hayashi et al., 2009; Wyndaele and De Wachter, 2003). Cytokines are thought to alter cell-cell communication by affecting gap-junctions in bladder smooth muscle and suburothelial myofibroblasts, in addition to altering voltage-gated K⁺ channel expression in bladder afferents (Hayashi et al., 2009; Heinrich et al., 2011; Wyndaele and De Wachter, 2003). IL-8 is a member of the chemokine family that plays an important role in attracting granulocytes, predominantly neutrophils to the site of inflammation (Petering et al., 1999). The biological action of IL-8 (also known as CXCL8) is activated when it binds to seven-membrane G-protein-coupled receptors named CXCR1 and CXCR2. IL-1 β is a potent mediator in response to infection and injury, and plays a vital role in a number of chronic and acute inflammatory diseases (Dinarello, 1998; Li et al., 2008). Majority of IL-1 β is synthesised by blood monocytes, but it is also produced by macrophages, dendritic cells and a variety of other cells in the body (Dinarello, 1996; Sasaki et al., 2002). A small amount of IL-1 β *in vivo* can cause fever, hypotension, release of adrenocorticotrophic hormone and production of cytokines which in turn lead to a number of inflammatory and immune responses (Li et al., 2008).

A recent study has suggested that inflammation may be a key factor in the development of bladder overactivity in chronic bladder ischemia detecting increased levels of pro-inflammatory cytokines including IL-8, IL-6 and TNF- α (Nomiya et al., 2012). Our data suggests that sustained release of inflammatory cytokines may be important in the development of the bladder overactivity and pain experienced by patients following intravesical treatment.

Nitric oxide

NO plays a major role in the bladder outlet region during micturition, inducing relaxation and preventing large increases in pressure during bladder contraction

(Bustamante et al., 2010). Along with acetylcholine, NO is released as a co-transmitter from parasympathetic nerves and exerts an inhibitory effect on contraction (Dokita et al., 1991). Recently the urothelium has been shown to be an alternative source of NO in the bladder; endothelial NO synthase (eNOS) has been identified in umbrella cells of the rat urothelium and stretch or muscarinic receptor stimulation has been shown to release NO from these cells in culture (Birder et al., 1998; Giglio et al., 2005). The function of urothelial NO is uncertain, but indirect evidence suggests a role in modulating sensory nerve activity (Birder et al., 1998; Giglio et al., 2005) and NO donors have recently been shown to inhibit afferent nerve activity (Aizawa et al., 2011). NO donors have been shown to inhibit urothelial contraction and inhibit pacemaker activity (Moro et al., 2012). There is also some evidence to suggest that NO can exert excitatory effects, such as in isolated mouse bladder it has been shown that NO can increase phasic contractile activity (Gillespie and Drake, 2004) and in precontracted human detrusor strips both relaxation and contraction responses to NO donors have been observed (Moon, 2002). Elevated levels of NO have been reported with inflammatory disease of the bladder including bacterial cystitis, IC and after BCG treatment for bladder cancer, and is thought to play a role in inflammation progression (Ehren et al., 1999; Logadottir et al., 2004; Lundberg et al., 1996). iNOS expression was shown to be localised in the urothelium of bladder cancer patients following BCG treatment (Koskela et al., 2008), while increased NOS expression and enhanced NO release from the bladder mucosa is also associated with cyclophosphamide-induced cystitis (Andersson et al., 2008). A recent study reported that inhibition of NOS prevents muscarinic and purinergic dysfunction and development of cyclophosphamide-induced cystitis in the rat (Aronsson et al., 2014). Elevated bladder wall NO production has been reported in patients with IC, with a concurrent increase in IL-6, -10 and -17A mRNA (Logadottir et al., 2014). Here we show an increase in NO release from urothelial cells 24 hours following MMC treatment which persists 1 week following first treatment but returns to normal 1 week following repeat treatment. These findings suggest that elevated NO release may temporarily contribute to the pain and alterations in bladder function experienced in patients following intravesical MMC treatment but persist the adverse effects by playing a role in inflammation progression which can be supported by observation of sustained inflammatory cytokine release even after repeat treatment in the present study.

Conclusions

Here we show that MMC treatment alters urothelial release of ATP, ACh and PGE₂ immediately, 24 hours and 1 week following treatment, but that release of these mediators except PGE₂ returns to normal 1 week following repeat treatment. In addition, enhancement in urothelial NO release 24 hours and 1 week following treatment was demonstrated without sustained release observed one week following repeat treatment. The results of this study therefore suggest that alterations in urothelial ATP (decrease), ACh (increase) and NO (increase) may directly contribute to adverse effects of MMC in the short-term but enhanced NO and induction of IL-8 which persist even after repeat treatment along with sustained increase in PGE₂ throughout the recovery period and following repeat treatment may contribute significantly in causing the pain and bladder dysfunction experienced by patients following intravesical MMC.

Chapter 6 - The effects of epirubicin on UROtsa urothelial cells

Introduction

In Chapter 5, the effects of MMC treatment on release of urothelial mediators, markers of inflammation and cell viability were assessed using the human urothelial cancer cell line, RT4. I previously used this cell line as a model of urothelial function to assess the effects of the cytotoxic drug doxorubicin during my Honours project (Kang et al., 2013). The results of these studies indicated that release of inflammatory cytokines was common to both drug treatments (doxorubicin and MMC) and may potentially play an important role in causing the side effects reported by patients. Interestingly, we found that while these agents alter release of urothelial mediators initially, there was partial or full recovery with time.

The RT4 cell line, while a useful model of urothelial mediator release is derived from malignant urothelial tissue, and so when a cell culture model derived from normal/non-cancer urothelial tissue (UROtsa cell line) became available we chose to use it for the study detailed in this chapter. In this study, UROtsa was used to assess the effects of epirubicin on normal urothelial function.

UROtsa cell line

The UROtsa cell line originated from a primary culture of normal human urothelium and was immortalised by SV-40 large T antigen. Following immortalisation, UROtsa cells did not acquire characteristics of neoplastic transformation (Petzoldt et al., 1995). UROtsa cells proliferate in serum-containing growth medium as a monolayer of cells with little evidence of uroepithelial differentiation. Under serum-free growth conditions, UROtsa cells exhibit raised, three-dimensional structures consisting of a stratified layer of cells that strongly resemble *in situ* urothelium (Rossi et al., 2001). An immunofluorescence study revealed expression of cytokeratin 7, which is normally expressed in a urothelial subtypes (e.g., umbrella, intermediate, and basal cells), confirming the urothelial origin of UROtsa cell line (Bakali et al., 2014). UROtsa have been used extensively to study mechanisms of carcinogenesis (Eblin et al., 2008; Johnen et al., 2013) and more recently as a model of urothelial function to assess release of urothelial mediators, regulation of release as well as studies to assess receptor expression (Bakali et al., 2014; Mansfield and Hughes, 2014e; Save et al., 2011; Save and Persson, 2010; Tyagi et al., 2006).

It has been demonstrated that UROtsa cells express the machinery for ACh synthesis (ChAT and CarAT) and muscarinic receptors with the rank order of $M_3 > M_2 > M_5 > M_1 = M_4$ (Arrighi et al., 2011). A study by Save & Persson (2010) demonstrated release of ATP and inflammatory cytokines (IL-8) from UROtsa cells and RT-PCR studies confirmed the presence of mRNA for P2Y₁, P2Y₂ and P2Y₁₁ purinoceptors in these cells. A further RT-PCR study by Save et al. (2011) reported transcriptions of adenosine receptor subtypes including A₁, A_{2A}, A_{2B} but not A₃ in UROtsa cells. Stretch-induced ATP release was demonstrated when UROtsa cells were treated with hypotonic Krebs solution and also an increase in ATP was observed with the P2Y agonists ADP and UTP (Mansfield and Hughes, 2014e). In addition, a recent study by Bakali et al. (2014) has shown expression of GPR55 cannabinoid receptor and also purinoceptors including P2X₁, P2X₂, P2Y₂ and P2Y₄ on UROtsa cells. When purinoceptors were activated with ATP, intracellular calcium increased significantly. Furthermore, radioligand binding study by Harmon, Porter & Porter (2005) has demonstrated β -adrenergic receptors (ARs) on UROtsa cells and when activated by the selective β -AR agonist isoprenaline enhanced protein levels for COX-2 and iNOS which are associated with inflammation. Hence, UROtsa may be a valuable *in vitro* model for studying the effect of chemotherapeutic agents on the function of 'normal' urothelium.

Aims

The aim of this study was to investigate the effects of epirubicin on basal and stretch-induced release of urothelial mediators (ATP, ACh and PGE₂) from UROtsa human urothelial cells and determine whether changes in urothelial function may provide an explanation for the urological adverse effects experienced by patients treated with intravesical epirubicin. The release of inflammatory cytokines and NO from urothelial cells after epirubicin treatment was also assessed to determine if inflammation may play a role in the adverse effects associated with treatment. In addition, recovery of urothelial function was assessed 1 week post-treatment. It was hypothesised, that while urothelial mediator release may change initially, there would be recovery over time but that a sustained increased in inflammatory cytokine release would be observed following epirubicin treatment, as seen in Chapter 5 with

MMC. This sequence of events was hypothesised to be common to chemotherapeutic drugs regardless of the *in vitro* model used.

Materials and Methods

Cell culture

The UROtsa cell line was received as a gift from Dr. Scott Garret at University of North Dakota. UROtsa were routinely cultured in 75cm² culture flasks (T75) using Dulbecco's modified eagle medium (DMEM) - low glucose culture medium supplemented with additional 1mg/ml glucose, 1% v/v penicillin-streptomycin and 5% v/v Fetal Bovine Serum (FBS). Cells were maintained at 37°C in 5% CO₂ and confluent cells (approx. 90%) were subcultured at a 1:4 ratio by detaching cells from the flask using a trypsin/EDTA solution.

Seeding densities of cell treatments

UROtsa cells were seeded in 24 or 96 well plates or T25 flasks at a density determined in initial optimisation experiments, see Table 6.1 below. Cells were incubated overnight at 37°C to allow cells to adhere.

Table 6.1: Optimum seeding density used for experiments with UROtsa

Timing of Endpoint Measurement	Seeding Density in 96 well plates	Seeding Density in 24 well plates	Seeding Density in T25 flasks
Immediate	0.2 x 10 ⁵ per well	1.2 x 10 ⁵ per well	1.6 x 10 ⁶ per well
24 hours after 1 st treatment	0.2 x 10 ⁵ per well	1.2 x 10 ⁵ per well	1.6 x 10 ⁶ per well
1 week after 1 st treatment	0.17 x 10 ⁵ per well	1.0 x 10 ⁵ per well	1.3 x 10 ⁶ per well
1 week after 2 nd treatment	0.13 x 10 ⁵ per well	0.8 x 10 ⁵ per well	1.1 x 10 ⁶ per well

Following attachment of UROtsa cells, culture medium was changed to serum-free DMEM-low glucose (supplemented with 1mg/ml glucose and 1% v/v penicillin/streptomycin) and incubated for further 24 hours to allow cells to differentiate. UROtsa were treated with epirubicin following 24 hour incubation in serum free medium.

Epirubicin treatment of urothelial cells

UROtsa cells were treated with epirubicin (0.0001, 0.001, 0.01, 0.1 and 1mg/ml) as described previously in MMC cell study. Epirubicin was diluted in serum-free DMEM culture medium and was allowed to equilibrate for 30 minutes at 37°C immediately before use. Serum-free DMEM culture medium was added to control cells. The plate was then incubated for one hour at 37°C in 5% CO₂.

To assess 24 hour and 1 week post-treatment effects of epirubicin on urothelial function, the same protocols were followed as described in the previous chapter, except for the following:

- (1) Concentration and duration of epirubicin treatment
 - 0.0001 or 0.01 mg/ml for 1 hour (24 hour post-treatment)
 - 0.001 mg/ml for 1 hour (1 week post-treatment)
 - epirubicin diluted in serum-free DMEM
- (2) After aspiration of drug, serum-free DMEM culture medium was added into each well.
- (3) During 1 week recovery period, culture medium was changed every three days.
- (4) Repeat treatments were also attempted in UROtsa, however cells did not survive repeat treatment and therefore endpoint analysis could not be conducted.

Assessment of cell viability - resazurin reduction assay

Cells were seeded in 96-well microtiter plates as previously described and treated with epirubicin (0-1 mg/ml) for 1 hour at 37°C. Then, resazurin reduction cell viability assay was performed immediately, 24 hour or 1 week after epirubicin treatment as described in Chapter 5, except the incubation period with resazurin was 1 hour at 37°C.

Mediator release from urothelial cells

Basal release

Immediately, 24-hour, or 1-week following 1 hour incubation with epirubicin, all solutions on the plate were aspirated and cells were washed twice with phosphate buffered saline (PBS) solution.

UROtsa cells were first incubated with serum-free DMEM culture medium for 15 minute incubation. After that, culture medium were aspirated and the cells were incubated for 15 minutes with isotonic solution (composition, mM: NaCl 130, KCl 5, CaCl₂ 1.5, MgCl₂ 1, NaHEPES 25, BSA 0.015 and glucose 5) at 37°C in 5% CO₂. Then, the solutions on the plate were aspirated and fresh isotonic solution was added into each well. After 10 minutes, the solutions in each well were collected into microfuge tubes and these are representative of basal release of urothelial mediators from epirubicin treated and control cells.

Hypo-osmotically stimulated release

Hypotonic solution (isotonic solution without NaCl) was added into each well. After 10 minutes incubation at 37°C, the solutions in each well were collected into microfuge tubes and these are representative of stimulated release of urothelial mediators from epirubicin treated and control cells.

Inflammatory cytokine and NO release from cell lines

To assess the late effects (24 hour post 1st treatment) of epirubicin on release of inflammatory cytokines and urothelial NO, cells were seeded in 25cm² flasks using conditions described above.

Then, procedures were followed by the same protocol used in the previous chapter, except for the following:

- (1) After treatment, serum-free DMEM culture medium was added
- (2) In additional experiments, where samples were also collected 1 week following treatment, media changes were performed every 3 days during the incubation period.

(The release of various mediators was measured using commercially available kits as described in “Assays for urothelial mediators, NO and inflammatory cytokines” section in Chapter 2)

Results

Effects of epirubicin on urothelial cell viability

Urothelial cell viability was significantly decreased in UROtsa cells treated with epirubicin $\geq 0.01\text{mg/ml}$ at all time points tested post-treatment. Complete cell death was observed in cells treated with 1mg/ml epirubicin compared to control cells at all time points (Figure 6.1a, b and c).

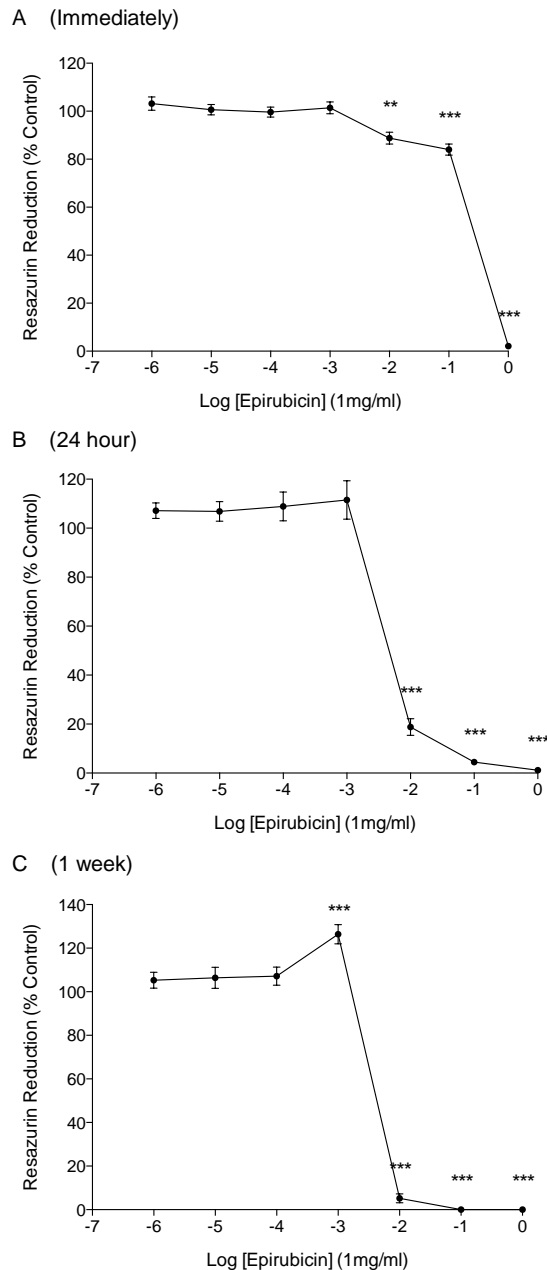


Figure 6.1: Effect of 1 hour epirubicin treatment on urothelial cell viability assessed by resazurin reduction immediately [A], 24 hour [B] and 1 week [C] after treatment. Data are expressed as means \pm SEM (N=6). **P<0.01 and ***P<0.001 compared to vehicle control cultures.

Effects of epirubicin on mediator release immediately post-treatment

Since, there was complete cell death observed at the clinical dose (1mg/ml) immediately following treatment, urothelial mediator release could not be measured in cells treated with this concentration.

UROtsa cells released ATP under basal and stimulated conditions, with a significant increase above basal levels of ATP in response to hypotonic stimulation observed in both vehicle treated control cells and cells treated with epirubicin (Figure 6.2a and b). Immediately following treatment, a significant increase (2-fold) in basal release of ATP from cells treated with 0.1mg/ml epirubicin was observed (Figure 6.2a). A significant increase (2-fold) in stimulated release of ATP was also observed from cells treated with the same concentration of epirubicin (Figure 6.2b).

Basal and stimulated release of ACh and PGE₂ were observed in both control and epirubicin treated UROtsa cells immediately following treatment (Figure 6.2c, d, e and f). There was a significant increase in release of both mediators in response to stretch when compared to basal levels. However, there was no significant change in release of ACh or PGE₂ from UROtsa cells immediately following treatment with epirubicin (Figure 6.2c, d, e and f).

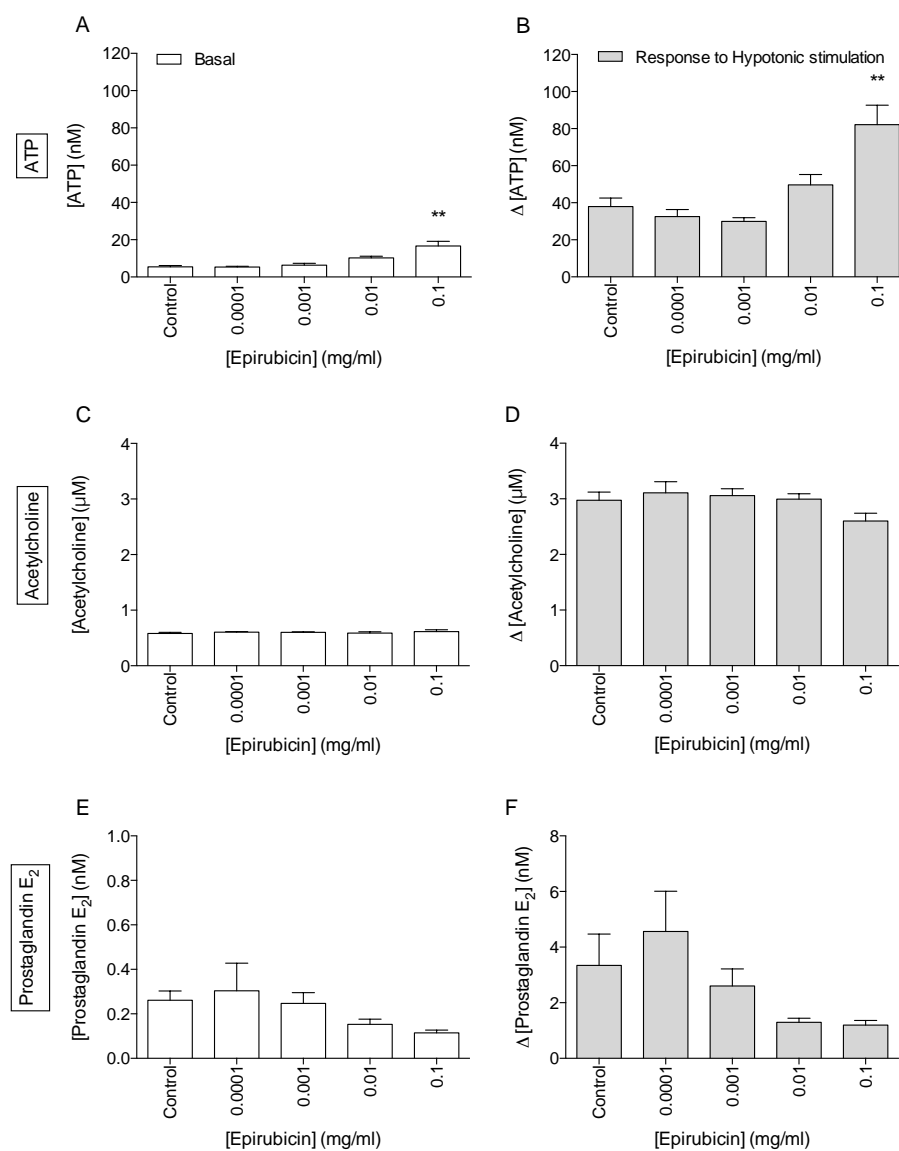


Figure 6.2: Immediate effects of 1 hour epirubicin treatment on basal extracellular ATP [A], acetylcholine [C] and prostaglandin E_2 [E] concentrations, and ATP [B], acetylcholine [D] and prostaglandin E_2 [F] response to hypo-osmotic stimulation. Data are expressed as means \pm SEM (N=6). **P<0.01 compared to vehicle control.

Effects of epirubicin on mediator release 24 hour post-treatment

Since urothelial ATP release was affected immediately after epirubicin treatment, the ability of the urothelium to recover was assessed. Release of urothelial mediators was measured 24 hour after epirubicin treatment with low epirubicin concentrations (≤ 0.01 mg/ml). As detailed in Chapter 5 previous studies have shown that only a small fraction of intravesical chemotherapeutic agents (doxorubicin and MMC) penetrates the urothelium, therefore lower concentrations of epirubicin are also likely to be more relevant.

Both basal and stimulated release of ATP from urothelial cells treated with 0.01mg/ml epirubicin increased significantly compared to vehicle-treated controls 24 hour following treatment (Figure 6.3a and b). While, basal release of ACh was significantly increased in cells treated with 0.01mg/ml epirubicin, stimulated release of ACh was significantly reduced at the same concentration in comparison to control, although stimulated ACh release was still greater than basal levels (Figure 6.3c and d). In contrast, both basal and stimulated release of PGE₂ remained unchanged compared to control cells 24 hour following epirubicin treatment (Figure 6.3e and f).

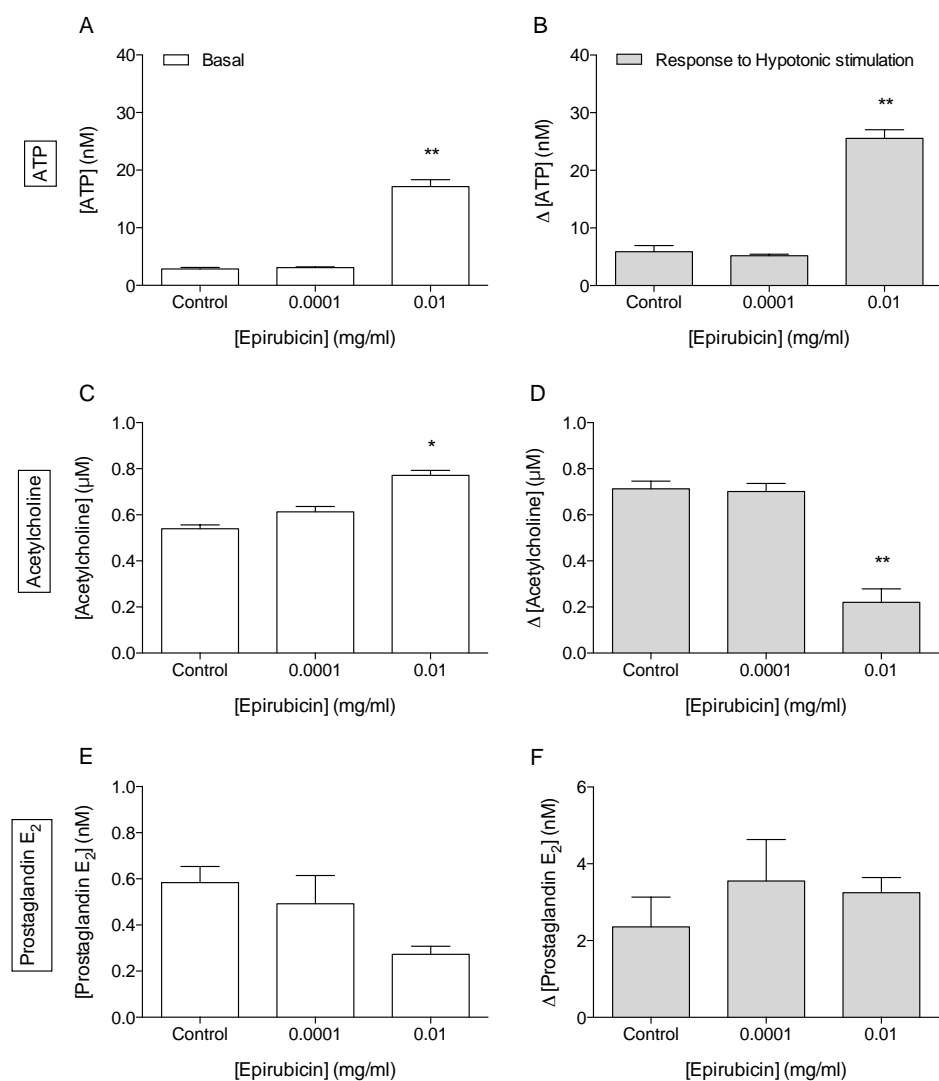


Figure 6.3: Twenty-four hour post-treatment effects of epirubicin on basal extracellular ATP [A], acetylcholine [C], prostaglandin E₂ [E] concentrations, and ATP [B], acetylcholine [D] and prostaglandin E₂ [F] response to hypo-osmotic stimulation. Data are expressed as means \pm SEM (N=6). *P<0.05 and **P<0.01 compared to vehicle control.

Effects of epirubicin on mediator release 1 week post-treatment

One week after treatment with 0.001mg/ml epirubicin, stimulated release of ATP was significantly decreased (Figure 6.4a and b), while stimulated release of ACh was significantly increased (Figure 6.4c and d) in treated cells compared to control cells. Both basal and stimulated release of PGE₂ remained unchanged compared to control cells 1 week following epirubicin treatment (Figure 6.4e and f). However, it must be noted that the PGE₂ increase over basal in response to stretch was no longer present at this timepoint, and likely reflects changes due to the prolonged period of serum starvation.

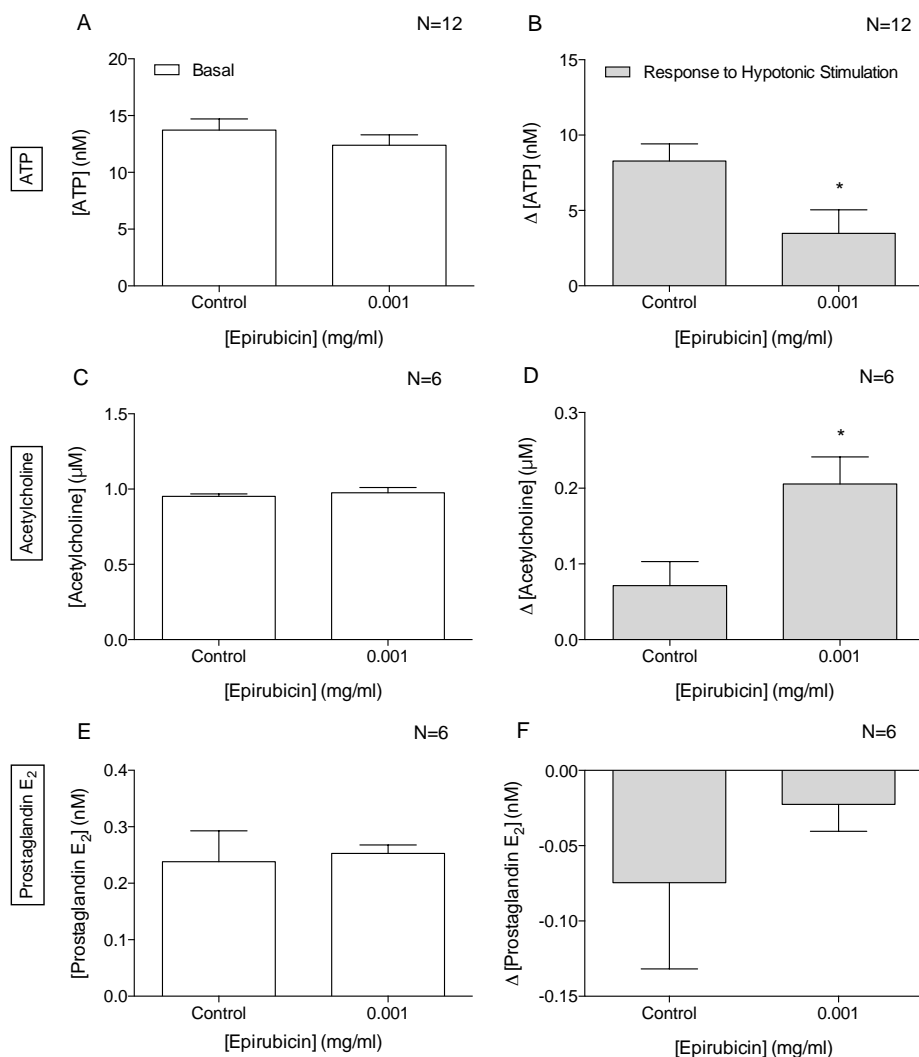


Figure 6.4: One week post-treatment effects of epirubicin on basal extracellular ATP [A], acetylcholine [C], prostaglandin E₂ [E] concentrations, and ATP [B], acetylcholine [D] and prostaglandin E₂ [F] response to hypo-osmotic stimulation. Data are expressed as means \pm SEM. *P<0.05 compared to vehicle control.

The effects of epirubicin on cell viability one week following repeat epirubicin treatment were assessed in this study. One week following repeat treatment, almost complete cell death ($8.2 \pm 1.2\%$, $N=6$) was observed in cells treated with 0.001mg/ml epirubicin. Therefore, it was not possible to measure the effects of epirubicin on urothelial mediator release following repeat treatment.

Effects of epirubicin on inflammatory cytokine and NO release

The presence of inflammatory cytokines IL-8, IL-1 β , IL-6, IL-10, IL-12p70 and TNF in urothelial cell incubation medium was tested 24 hour and 1 week after epirubicin treatment as a measure of urothelial inflammation. IL-1 β , IL-6, IL-10, IL-12p70 and TNF were not detected in control or treated samples at any timepoint tested. IL-6 was secreted by untreated urothelial cells at 24 hour, while urothelial cells treated with epirubicin (0.01mg/ml) showed a 70-fold increase in release ($53.24 \pm 5.02\text{ pg/ml}$ control vs. $3511 \pm 494\text{ pg/ml}$ treated, $P<0.01$) (Figure 6.5a). IL-8 was also secreted by untreated urothelial cells at 24 hour, while urothelial cells treated with epirubicin (0.01mg/ml) showed a 5-fold increase in release of IL-8 ($292 \pm 23\text{ pg/ml}$ control vs. $1519 \pm 89\text{ pg/ml}$ treated, $P<0.01$) (Figure 6.5c). IL-6 (8-fold increase, $P<0.001$) and IL-8 (3-fold increase, $P<0.001$) secretions were also significantly increased compared to controls 1 week after epirubicin treatment (Figure 6.5b and d).

Metabolites of NO (nitrate and nitrite) were measured in incubation medium to determine the effects of epirubicin on urothelial NO release. NO was released by untreated UROtsa cells at 24 hour, with a significant increase observed from UROtsa cells treated with epirubicin (0.01mg/ml) (Figure 6.6a), but 1 week after epirubicin treatment no significant differences could be detected compared to control (Figure 6.6b).

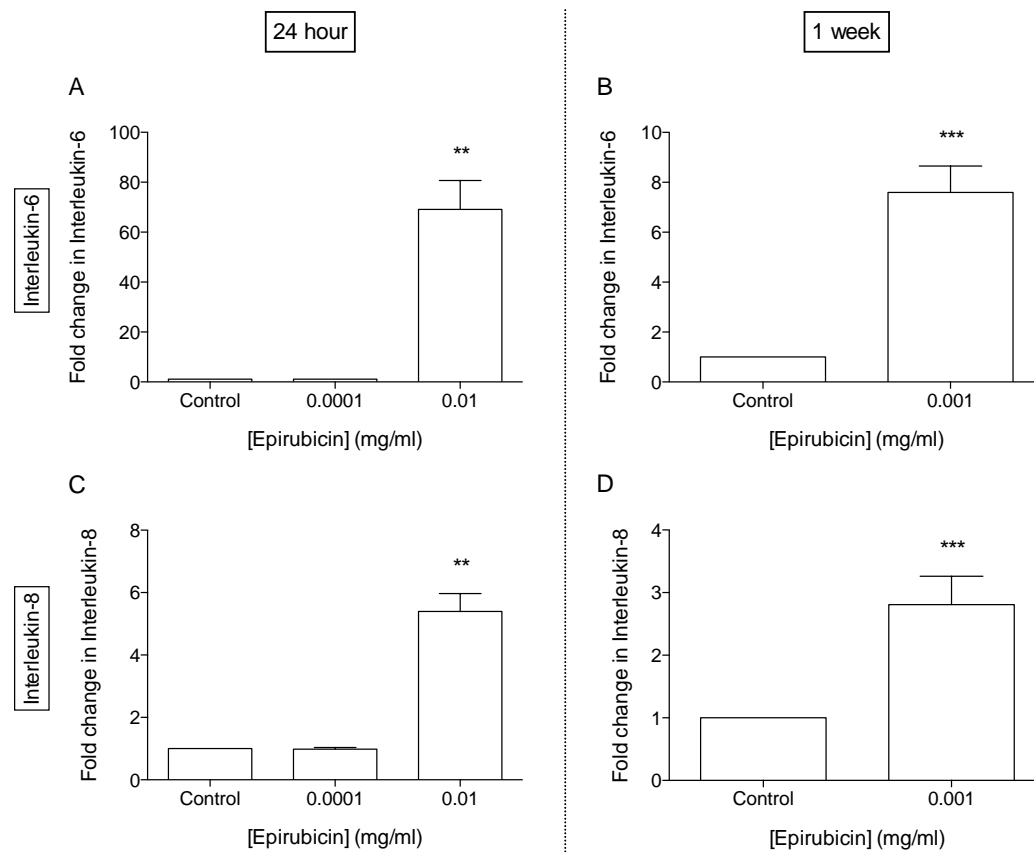


Figure 6.5: Interleukin-6 secreted from control and epirubicin treated urothelial cells 24 hour [A] and 1 week [B] after treatment. Interleukin-8 secreted from control and epirubicin treated urothelial cells 24 hour [C] and 1 week [D] after treatment. Data are expressed as means \pm SEM (N=6). **P<0.01 and ***P<0.001 compared to vehicle control.

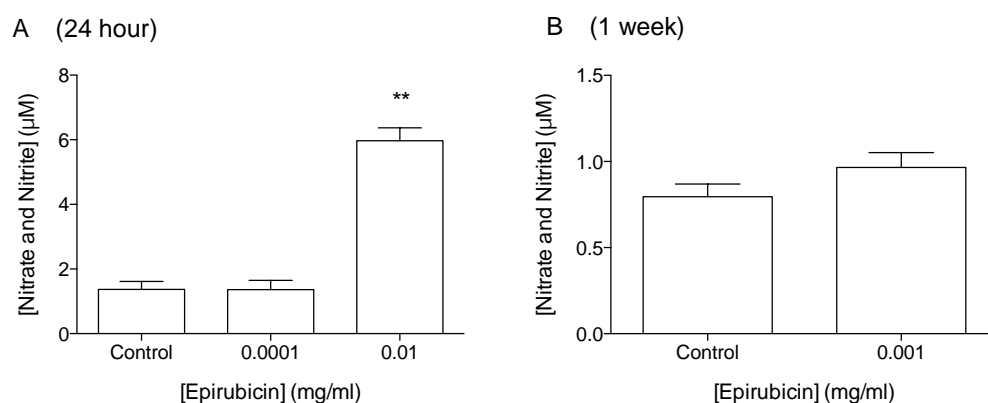


Figure 6.6: Effect of epirubicin on metabolites of nitric oxide (nitrate and nitrite) 24 hour [A] and 1 week [B] post-treatment. Data are expressed as means \pm SEM (N=6). **P<0.01 compared to vehicle control.

Discussion

ATP

Here we report enhanced release of both basal and stimulated ATP immediately after epirubicin treatment at 0.1mg/ml, which was still evident 24 hours following treatment. In Chapter 3, the close association of enhanced ATP release with bladder disorders such as OAB and IC/PBS, which are also characterised by symptoms common in patients treated with intravesical epirubicin, has been discussed in detail. Therefore, increased urothelial ATP release observed in this study could directly contribute to the pain and bladder overactivity reported in patients treated with intravesical epirubicin. In contrast, doxorubicin has been shown to have inhibitory effects on urothelial ATP release (Kang et al., 2013) as did MMC (see Chapter 5). Interestingly, a decrease in stimulated ATP release was observed one week following epirubicin treatment. This may indicate time-dependent rather drug specific effects. How a decrease in urothelial ATP release could contribute to some of the reported side effects has been discussed in detail in Chapter 5.

Acetylcholine

A recent study reported that UROtsa cells express the cellular machinery required for synthesis of ACh, as well as expressing all 5 muscarinic receptors (Arrighi et al. 2011). Here we found that the cells release ACh under basal conditions with a significant increase in release in response to stretch. The potential excitatory effect of urothelial-derived ACh on the micturition reflex has been discussed previously in Chapter 4. Thus, enhanced release of basal urothelial ACh 24 hours following epirubicin treatment may activate muscarinic receptors in the bladder which can potentially cause overactivity of the bladder reported in patients treated with intravesical epirubicin. The observed increase in ACh levels may be due to the effects of epirubicin influencing the release of other mediators which play a role in controlling ACh release (as mentioned previously in this thesis, ACh and ATP are co-released from parasympathetic nerves and NO can facilitate vesicular release of ACh and ATP) or due to changes in ACh breakdown or reuptake mechanisms. An increase in stimulated release of urothelial ACh one week following epirubicin was

observed and this may also contribute to bladder overactivity. However, as it was mentioned for ATP, this may also be time-dependent rather drug specific effects.

Since there is a growing consensus that ACh released from the urothelium has a role in excitatory mechanism of bladder function, it would be expected that a decrease in ACh release may attenuate overactivity of the bladder, thus not explaining the adverse effects of intravesical epirubicin. However, the decrease in extracellular ACh concentration caused by hypotonic stimulation observed 24 hours following epirubicin treatment in this study may cause an imbalance in cholinergic systems in the bladder, potentially contributing to bladder dysfunction. Its implication in OAB conditions is unknown. This may be due to increased ACh breakdown rather than a decrease in release, which is supported by reports that epirubicin has been used to differentiate neuroblastoma cells and was shown to increase acetylcholine esterase (AChE) activity (Rocchi et al., 1987). However, a recent study reported that systemic chemotherapy for gastric adenocarcinoma which included epirubicin resulted in decreased AChE activity in gastric tissue possibly contributing to alteration in neuro-muscular gastric function in cancer patients receiving this treatment (Sung et al., 2012). The decrease in AChE activity report by Sung et al. may also be due to the other chemotherapeutic drugs used as part of the treatment regimen rather than a specific effect of epirubicin.

Prostaglandin E₂

Epirubicin had no effect on either basal or stimulated release of PGE₂ from UROtsa cells immediately, 24 hours and 1 week post-treatment. Therefore, unlike for doxorubicin (Kang et al., 2013) and MMC (see Chapter 5), PGE₂ does not appear to play a role in causing the adverse effects reported in patients treated with intravesical epirubicin. In both pretreated and untreated (control) cells, an increase in ATP and ACh release over basal levels in response to stretch was observed one week following epirubicin treatment. However, increased PGE₂ release in response to hypotonic stimulation was no longer present in control or pretreated cells at this timepoint. This may be due to the effects of long-term serum deprivation on cell function. It is well established that serum starvation in cells for several days can induce G0/G1 cell cycle arrest. A study by Khammanit et al. (2008) using canine dermal fibroblast cells has demonstrated that short periods of serum starvation (24 to

72 hours) increased the proportion of cells at the G0/G1 phase (88.4 to 90.9%) compared to controls (73.6%). Also, a recent study using sheep granulosa cells reported significantly increased G0/G1 phase cells after serum starvation for 24, 48 and 72 hours when compared to normal growing cells ($P < 0.05$). A significant apoptosis of granulosa cells occurred when serum starved for 72 hours ($P < 0.05$) (Sadeghian-Nodoushan et al., 2014). In addition, a study by Hasan et al. (1999) using Chinese hamster V79 cells has demonstrated that serum starvation led to growth arrest, rounding up of cells and the appearance of new protein kinase C (PKC)- α and p53 bands on Western blots. Prolonged incubation (≥ 48 hours) in serum-starved medium caused cell detachment and death. It is known that serum-starved conditions result in expression of structural features of differentiated urothelium (Rossi et al., 2001). However, the effects of longer period serum starvation such as one week on cell function are not well known. However, a study by Zhang et al. (2006) which serum starved (0.5% serum) human diploid fibroblasts for about 70 days, has found that the cells were transformed (became round and had more than one nucleolus) and also led to cell chromosomal instability (such as abnormal karyotype including aneuploidy and structural aberrations and high telomerase activities). The serum-starvation for one week in this study has not resulted in a decrease in cell viability in control cells, thus UROtsa cells may not undergo apoptosis during this period, but instead may potentially lead to changes in cell function such as affecting stimulated PGE₂ release. Therefore, determining the effects of long-term serum starvation on urothelial function would be beneficial in interpreting the recovery results (1 week post-treatment) from studies using UROtsa cells that differentiate in serum-free growth conditions.

Nitric oxide

An increase in NO release from UROtsa cells was observed 24 hours following epirubicin treatment. This is consistent with the effects of MMC on RT4 urothelial cells however the levels returned to normal 1 week following epirubicin treatment unlike in MMC treated cells where the changes in NO release were still evident 1 week later. Changes in NO release did not remain following repeat MMC treatment. These finding suggests that an initial increase in NO release is common to MMC and epirubicin treatment, but recovery to normal levels is time dependent. Longer time

to recovery may also reflect why side-effects are more commonly reported following MMC treatment compared to other intravesical drugs (Shelley et al., 2012). The potential for enhanced NO release to contribute to pain and overactivity has been discussed in Chapter 5. Several studies have assessed the impact of anthracyclines (including doxorubicin and epirubicin) on NO signalling pathways, in particular in relation to the cardiotoxicity associated with these agents. There is evidence that exposure to these drugs can increase myocardial iNOS expression resulting in enhanced NO production (Pacher et al., 2003; Weinstein et al., 2000).

Inflammatory cytokine

In the present study, when the effects of epirubicin on release of inflammatory cytokines from UROtsa cells were assessed, increases in IL-8 and IL-6 were observed 24 hours following treatment and both inflammatory cytokines were still elevated 1 week after treatment. These findings are similar to the observation made in Chapter 5 where an increase in IL-8 from RT4 urothelial cells 24 hours and 1 week after initial MMC treatment and also 1 week after repeat MMC treatment were demonstrated. A recent study by our group also reported that doxorubicin treatment induces release of IL-8 and IL-1 β from RT4 cells 24 hours following treatment (Kang et al., 2013). Thus, it can be postulated that the induction of inflammatory cytokines and their persistent release are common to chemotherapeutic agents doxorubicin, MMC and epirubicin and may play a significant role in causing the bladder overactivity and pain experienced by patients following intravesical chemotherapy (see Chapter 5 for detailed discussion of how enhanced release of inflammatory cytokine can lead to overactivity and pain).

In Chapter 5, the function of IL-8 and IL-1 β in immune system has been discussed. There is evidence suggesting that IL-6 may play an important role in the generation and propagation of chronic inflammation. IL-6 trans-signalling via soluble IL-6 receptors increases the expression of endothelial leukocyte adhesion molecules (VCAM-1, ICAM-1), promoting leukocyte accumulation (Barnes et al., 2011; Hurst et al., 2001; Jones et al., 2005; Rose-John, 2012). Also, IL-6 may have a role in promoting neutrophil apoptosis and thus the resolution of acute (nonspecific) inflammation (Afford et al., 1992; McLoughlin et al., 2003). However, an antiapoptotic effect of IL-6 on neutrophils has been also reported (Colotta et al.,

1992). In addition, IL-6 reportedly rescues T cells from apoptosis, which promotes a chronic inflammatory cell infiltrate (Curnow et al., 2004; Narimatsu et al., 2001; Takeda et al., 1998; Teague et al., 1997). IL-6 trans-signalling also promotes the release of IL-6 from fibroblasts and endothelial cells in a positive autocrine feedback system (Barnes et al., 2011). It should be noted that the increase in IL-8 release following MMC treatment was much greater (326-fold) compared to that for the other agents tested (5-fold for epirubicin and 5.5-fold for doxorubicin) which again may reflect the greater incidence of urological adverse effects following MMC treatment.

In addition, decreases in stimulated release of ATP reported here may be due to the effects of inflammatory cytokine release by epirubicin treatment, since a study by Mansfield & Hughes (2014a) demonstrated that exposing human urothelial cells to inflammatory mediators (histamine and serotonin) resulted in reduced stretch-induced release of ATP. However, the increased ATP release observed in this study could also be due to inflammation since there is a study that demonstrated increased ($P<0.01$) extracellular ATP levels when UROtsa cells were infected with uropathogenic *Escherichia coli* IA2 strain (Save and Persson, 2010).

Furthermore, when the effects of epirubicin treatment on viability of UROtsa cells were assessed, a concentration-dependent decrease in urothelial cell viability was observed with significant changes detected in cells treated with epirubicin $\geq 0.01\text{mg/ml}$ immediately, 24 hours and 1 week following treatment. However, 1 week following repeat treatment almost complete cell death ($8.2\pm1.2\%$) was demonstrated in cells treated with a 1000-fold lower than clinical concentration of epirubicin. It can be postulated that significant cytotoxicity of epirubicin on the urothelium by repeated epirubicin treatment may be due to sustained release of inflammatory cytokines observed in this study which can induce inflammation of the bladder, causing the urothelium to be more susceptible to the cytotoxic effects of epirubicin or may reflect the prolonged period in serum-free culture.

Conclusions

The present study has shown that epirubicin treatment alters urothelial release of ATP and ACh immediately, 24 hours and 1 week following treatment. In addition,

enhancement in urothelial NO release 24 hours, but not 1 week, after treatment was observed.

In conclusion, the results of our study suggest that while it is evident that alterations in urothelial mediator release (ATP, ACh, and NO) can contribute both temporarily and long-term to local adverse effects experienced by patients treated with epirubicin, it appears that epirubicin exerts adverse effects primarily through induction of urothelial inflammatory cytokine release and their sustained release (one week following treatment), which has been reported to be strongly associated with development of bladder dysfunction such as pain and overactivity, possibly causing the urothelium to be more vulnerable to cytotoxic effects of epirubicin in long-term (evident by significant cell death one week following repeat treatment).

Interestingly, the release of inflammatory cytokines is common to all of the chemotherapeutic agents tested. Future pharmacological and physiological studies are necessary to confirm if our conclusions regarding the role of inflammation are valid and whether interventions to reduce inflammation could potentially improve the tolerability of these agents without affecting cytotoxic efficacy on cancer cells.

Chapter 7 - General discussion

The aim of this thesis was to explain the urinary adverse effects noted by patients following intravesical treatment with doxorubicin, MMC and epirubicin. Hence, a number of studies, utilising a variety of techniques and *in vitro* models, were conducted. Subsequently, throughout the thesis, various changes in the local bladder mechanisms, that are known to be involved in maintaining normal bladder function, were identified after treatment with these agents. In addition, age-related changes in bladder function were evident when the findings of young and aged porcine tissues were compared. They were discussed in detail in Chapter 3, hence will not be further mentioned in this final discussion.

Studies using tissues dissected from bladders of aged pigs demonstrated a number of alterations in bladder function following cytotoxic drug treatments and some similarities and differences were observed among the three cytotoxic agents (Table 7.1). While doxorubicin treatment had no effect on integrity of the urothelium, both MMC and epirubicin treatment resulted in urothelial thinning. Contractile responses of urothelial/LP strips to muscarinic stimulation were not affected by epirubicin treatment, however following doxorubicin and MMC treatment the responses were enhanced and inhibited, respectively. The effect of UDIF on detrusor contractility was unaltered following doxorubicin treatment, where the presence of an intact urothelium/LP depressed maximum contractions of the detrusor muscle to carbachol. However, this UDIF effect was abolished by both MMC and epirubicin treatment. Also, contractile responses of the detrusor muscle strips to muscarinic stimulation were enhanced following doxorubicin treatment, while the responses were inhibited by both MMC and epirubicin treatment. In addition, relaxation responses of the detrusor muscle to isoprenaline were reduced by MMC treatment, however both doxorubicin and epirubicin treatment demonstrated no effect on detrusor relaxation. Furthermore, neurogenic detrusor contractility was inhibited following doxorubicin and MMC treatment, however epirubicin treatment had no effect on neurogenic responses of the detrusor muscle. It was demonstrated in tissue studies, that MMC showed greater toxicity on bladder functions compared to doxorubicin and epirubicin, this is consistent with the high incidence of reported local side effects in patients following intravesical MMC. However, it is difficult to conclude whether epirubicin demonstrated lesser bladder toxicity than doxorubicin. These changes in bladder function appear to drug specific and how they might contribute local side effects in patients has been discussed in detail in chapters 3 and 4.

Table 7.1: The effects of doxorubicin, MMC and epirubicin on tissue responses of aged porcine bladders.

	DXR	MMC	EPR
Urothelial thickness	↔	↓	↓
Urothelial contractility (muscarinic)	↑	↓	↔
UDIF effect	↔	Abolished	Abolished
Detrusor contractility (muscarinic)	↑	↓	↓
Detrusor relaxation (adrenergic)	↔	↓	↔
Detrusor neurogenic response (EFS)	↓	↓	↔

Doxorubicin (DXR); Epirubicin (EPR)

Increase ↑; Decrease ↓; Unchanged ↔

The release of urothelial mediators following cytotoxic drug treatments was measured in both tissues and cell cultures, with recovery also assessed in cell culture experiments. Immediately following doxorubicin treatment in urothelial/LP strips from aged bladders, increase in stretch-induced ATP release was demonstrated, while ACh and PGE₂ release was not affected. In contrast, immediately following doxorubicin treatment in urothelial cells, abolition of stretch-induced ATP release, increased basal ACh release, decreased stretch-induced ACh, and increased stretch-induced PGE₂ release were observed. Twenty four hours following doxorubicin treatment in urothelial cultures, partial recovery of ATP release was shown, but without observing recovery, basal ACh and PGE₂ release remained enhanced and stretch-induced ACh remained depressed (Table 7.2).

Epirubicin caused decrease in stretch-induced ATP release and increase in basal ACh release, but had no effect on PGE₂ release immediately following treatment in urothelial/LP strips from aged bladders. Similarly, PGE₂ release was also unaffected in urothelial cells immediately after epirubicin treatment. However, both basal and stretch-induced ATP release were increased and ACh release was unaltered immediately following epirubicin treatment in cells. One week following treatment, recovery of basal ATP release and decrease in stretch-induced ATP release were demonstrated. Recovery of basal ACh release, and increase in stretch-induced ACh release were observed 1 week following epirubicin treatment in cells, while release of PGE₂ was unaffected (Table 7.2).

Immediately following MMC treatment, decrease in stretch-induced ATP release was demonstrated in both tissues and cells, however the release of ACh and PGE₂ differed when compared between two *in vitro* models. Increase in basal and stretch-induced ACh release was observed in tissues and cells. In addition, increase and decrease in the release of basal PGE₂ was demonstrated in tissues and cells, respectively. Recovery of ATP and ACh release, partial recovery of basal PGE₂ release, and increase in stretch-induced PGE₂ release were observed one week following repeat MMC treatment (Table 7.2). The differences between tissue and cell responses in mediator release may be due to the different cell types present. A study by Cheng et al. (2011) using porcine bladders demonstrated that cultures of urothelial cells, myofibroblasts and also detrusor muscle cells all release ATP in both basal and stretched conditions and may account for some of the differences observed here. Given that much of the changes in urothelial mediator release recover at least in part over time, suggests that in the long-term mediator release may not play a major contributing role in the ongoing side-effects experienced by patients.

Table 7.2: The effects of doxorubicin, MMC and epirubicin on urothelial mediator and cytokine release from urothelial/LP tissues and urothelial cell cultures.

	Urothelium/LP			Urothelial Cultures			
	ATP	ACh	PGE ₂	ATP	ACh	PGE ₂	Cytokines
DXR	↑↑	⇔	⇔	PR	↑ & ↓	↑↑	↑↑
MMC	↓↓	↑↑	↑	R	R	PR & ↑↑	↑↑
EPR	↓↓	↑	⇔	R & ↓	R & ↑	⇔	↑↑

Doxorubicin (DXR); Epirubicin (EPR)

Basal release (blue colour); Stretch-induced release (red colour)

Recovery (R); Partial recovery (PR); Increase ↑; Decrease ↓; Unchanged ⇔

One of the major findings of this study was the evidence of inflammation in the urothelium following treatment with all three agents, observed by induction of cytokines and their sustained release without recovery while other mediators that were also affected by these drugs observed partial or full recoveries (Table 7.2). Therefore, this final discussion will focus on how inflammation is associated with pathophysiology of various bladder dysfunctions and may explain the local adverse effects associated with intravesical chemotherapy.

Inflammation and urothelial abnormalities

The fundamental function of the urothelium is to form a high-resistance barrier to ion, solute and water flux, and pathogens (Birder, 2010). Three components that seem to be responsible for this property are tight junctions, plaques and the glycosaminoglycan (GAG) layer. The urothelial plaques or asymmetric unit membrane (AUM) are constructed from uroplakins (UPs), which consist of four major forms including UPIa and Ib, UPII and UPIII (Garcia-Espana et al., 2006). Tight junctions, which are localised between the umbrella cells, are composed of multiple proteins that include occludins and claudins. These proteins, together with uroplakins, assemble into hexagonal plaques, contributing to the urothelial barrier function (Hicks, 1975; Liang et al., 2001). The GAG layer is composed of negatively charged sulfated polysaccharides which are bound to core proteins that also have a negative charge and this is believed to be due to N-terminal sialic moieties (Parsons, 2011). Together they form a proteoglycan layer that binds water into a gel form via a process known as electrostatic entrapment. This results in a buffer between irritants in urine and the urothelial cell membrane, acting as the first line of defense. The GAGs associated with the bladders includes heparan sulfate, chondroitin sulfate, dermatan sulfate and hyaluronic acid (Buckley et al., 1996; Hurst and Zebrowski, 1994; Janssen et al., 2013). Therefore, any disruption to the components of this permeability barrier may lead to increased infiltration into the bladder wall and exposure of interstitial fluid to the potentially harmful contents of urine (Hurst et al., 1996; Parsons, 2011; Parsons et al., 2005; Parsons and Hurst, 1990). UPIII gene knockout mice in a study by Hu et al. (2000) demonstrated overexpression of UPIa, UPIb and UPII, defective glycosylation, and abnormal targeting of UPIb, resulting in small plaques and urothelial leakage. Also, a study by Janssen et al. (2013) reported an increased permeability of the urothelium after removing the GAG layer.

There is a growing body of evidence to support the conclusion that abnormalities in the urothelium may be involved in pathophysiology of functional bladder disorders such as IC/PBS and OAB, which share LUT symptoms that are also commonly reported in patients treated intravesically with three agents (Keay et al., 2014). Abnormalities such as an increased permeability of the urothelium are common in these bladder conditions (Hauser et al., 2008; Hurst et al., 1996; Parsons, 2011; Parsons et al., 1998; Parsons et al., 1991). Especially in patients with IC/PBS, this may be due to a perturbation to the components of urothelial barrier function

including denudation and tears in the bladder urothelium (Johansson and Fall, 1990; Ratliff et al., 1994; Tomaszewski et al., 2001), and/or thinning of the bladder urothelium to 1-2 cell layers thick (Tomaszewski et al., 2001). In microscopic studies, bladder urothelial abnormalities were confirmed in IC/PBS patients with disruption of the AUM and bladder urothelial junctions of the umbrella cells (Elbadawi and Light, 1996). Also, the urothelial mucin layer has been shown to be abnormal in IC/PBS (Hanno, 2002; Tomaszewski et al., 2001). In addition, a study by Parsons (2002) has demonstrated reduced formation of GAGs and one of their metabolite, uronic acid in urine of IC patients in comparison to control group. As it was mentioned previously in Chapter 5, inflammation is also closely associated with these conditions. However, it is unclear whether the compromised barrier function, noted by various abnormalities in the urothelium, causes the related inflammation or is a subsequent result of inflammation.

The results in this thesis also demonstrated abnormalities in urothelial thickness following MMC or EPI treatment as well as indication of inflammatory responses in urothelium cultures after treatment with the three agents. A correlation between inflammation and urothelial abnormalities was shown in a study by Shie & Kuo (2011). This study revealed significantly decreased cell proliferation, lower expression of junction protein E-cadherin and increased number of apoptotic cells in bladder urothelial tissues from IC/PBS patients compared to control bladder tissue, indicating greater damage level of the urothelium in patients with IC/PBS compared to control. Activation of mast cell, which is an important source of several inflammatory mediators including proteases and vasoactive amines such as histamine (Zhang et al., 2012b), was also significantly increased in tissues from IC/PBS patients and this mast cell activation significantly correlated with the apoptotic cell numbers. A further study by this group demonstrated that the levels of inflammatory molecules and pro-apoptotic proteins were significantly increased in the IC/PBS bladder, and phospho-p38 and TUNEL double staining indicated that inflammatory and apoptotic events coexisted in these patients. These results were confirmed by immunoblotting and suggested that the tissue damage and abnormal urothelium in the IC/PBS patients could result from upregulation of inflammatory mediators, including p38 mitogen-activated protein kinase and TNF- α . The in vitro analysis also showed that the apoptotic process could be induced by TNF- α treatment and anisomycin stimulation in normal urothelial cells (Shie et al., 2012). Thus, it can be

postulated that the urothelial cell damage caused by the three cytotoxic agents such as decreased urothelial thickness and cell viability, may be a secondary effect of inflammation where increased release of inflammatory cytokines observed after treatment could trigger the damage to the urothelium.

One of the widely accepted mechanisms of bladder toxicity of the three intravesical agents tested here is ROS production, which can be cytotoxic to both cancer and normal cells and these mechanisms have been discussed in detail in Chapter 1. Several enzymes are capable of producing ROS but the most important of these is NADPH oxidase (NOX) (Hancock et al., 2001). NOX is predominantly found in white blood cells such as neutrophils (Mittal et al., 2014) but has been also found to be present in cells that have no role in host defense such as fibroblasts (Meier et al., 1991), mesangial cells (Radeke et al., 1991), endothelial cells (Jones et al., 1996), osteoclasts (Steinbeck et al., 1994) and chondrocytes (Hiran et al., 1997). The ROS serve as both a signaling molecule and a mediator of inflammation at low concentrations (exact concentrations still remain to be determined) but if excessive and unchecked, they are injurious to cells at high concentrations because they can oxidize protein and lipid cellular constituents and damage the DNA (Droge, 2002; Mittal et al., 2014; Thannickal and Fanburg, 2000).

There are several studies supporting that inflammation could induce ROS production. A previous study by Meier et al. (1989) using primary cell cultures of human fibroblasts collected from healthy skin has demonstrated that treatment of these cells with cytokines IL-1 and TNF- α released hydrogen peroxide (H₂O₂) that increased with increasing treatment durations and concentrations. Also, a study using articular chondrocyte cells isolated from rabbits has shown that exposure to IL-1, TNF- α and interferon- γ (IFN- γ) for a period of 18 to 20 hour increased production of H₂O₂ compared to control (Tiku et al., 1990). Treatment of bovine articular chondrocytes with TNF- α also induced production of ROS (Lo and Cruz, 1995). A more recent study by Yang et al. (2007) demonstrated a time- and dose-dependent increase in both intracellular and extracellular ROS production from human RPE (retinal pigment epithelium) cells (isolated from donor eyes) in response to IL-1 β , TNF- α and IFN- γ stimulation. In addition, there is evidence showing that Rac1, which is an important cytosolic subunit that is required for activation of one of the NADPH oxidase homologs NOX2, can be activated by a variety of inflammatory stimuli

including IL-1 β (Monaghan-Benson and Burridge, 2009). Furthermore, a study by Bulua et al. (2011) has shown that mitochondrial-derived ROS (MtROS) can contribute to LPS-mediated production of pro-inflammatory cytokines IL-1 β , IL-6, and TNF- α , suggesting that the ROS produced in response to inflammation can further enhance cytokine release that may lead to much higher production of ROS.

This may explain the mechanism of bladder toxicity of the three intravesical agents whereby doxorubicin, epirubicin and MMC elicit inflammatory response to cause urothelial damage via oxidative stress, resulting in loss of the barrier function. The loss of barrier function can then lead to exposure of the bladder wall to infiltration of urinary constituents like potassium that can depolarize muscle and nerve cells, inflame tissues, degranulate mast cells, and cascade to the development of LUT symptoms (Parsons, 2011). In addition, disorders in normal glycosylation of UPs lead to abnormal epithelial adhesion, leakage of the urinary tract and spread of *Escherichia coli* (*E. coli*) infection, potentially exacerbating inflammation (Taganna et al., 2011). A study by Soler et al. (2008) has demonstrated that damage to urothelial barrier function by intravesical instillation of protamine sulfate in rats led to inflammation but in rats that underwent bilateral nephrectomy to prohibit urine production no inflammation resulted, supporting that the increased permeability of the barrier function to noxious substances in the urine can aggravate initial inflammation induced by intravesical agents and lead to chronic inflammation which is thought to be a potential mechanism of pathogenesis for bladder disorders (Erickson et al., 2002; Ogawa et al., 2010; Saini et al., 2008).

Above, it was proposed that a major mechanism of bladder toxicity by three intravesical agents may be their ability to elicit inflammation causing the disruption to urothelial barrier function, via excessive production of ROS, which can subsequently lead to a cascade of effects on bladder function. This theory was based on the findings of this thesis obtained from *in vitro* models, human urothelial cell lines and porcine bladder tissues. Thus, the clinical relevance of this concept needs to be considered. The viability of each *in vitro* model in terms of their physiological similarities to *in vivo* bladders such as receptor expressions and mediator release that are pivotal in regulation of storage and voiding function have been discussed in detail in respective chapters. However, resemblance in urothelial barrier components of these models has not been mentioned previously. Lobban and his colleagues (1998)

at the Cancer Medicine Research Unit in St. James's University Hospital (Leeds, United Kingdom) cloned cDNA sequence for human uroplakins UPIa, UPIb, UPII and UPIII, and used them to investigate uroplakin transcription by normal and neoplastic urothelial cells. It was found that normal human urothelial cell line obtained from patients with no history of urothelial dysplasia or malignancy as well as neoplastic cell line RT4 expressed mRNA for all four uroplakins. A freeze fracture analysis by Rossi et al. (2001) has demonstrated that the normal human urothelial UROtsa cell line possessed the tight-junction sealing strands and gap junctions.

A previous study by Wu et al. (1994) reported that the AUMs, which form the apical plaques of the urothelium, appear morphologically similar in human and pig urinary bladders bearing crystalline, hexagonal arrays of uroplakin and immunoblotting confirmed the presence of four uroplakins in AUMs of these species. Also, well differentiated superficial cells on the surface of the urothelium and intact tight junctions between cells have been reported in pig urinary bladders (Kos et al., 2006). In addition, immunohistochemistry experiments by Janssen and his colleagues (2010) reported the presence of a GAG layer in the RT4 cell line and GAG isolation experiments showed that the GAG layer produced by these cells contains heparan sulfate, chondroitin sulfate, dermatan sulfate in respectively decreasing concentrations and this distribution of GAGs is similar to those produced by the human bladder urothelium. Furthermore, a recent study by the same group has shown the presence of GAG in both human and porcine bladder biopsies and no differences were observed in GAG expression between these mammalian species. GAG isolation has revealed that the luminal side of the urothelium contained predominantly chondroitin sulfate and low amounts of heparan sulfate. Similar concentrations of chondroitin sulfate and heparan sulfate were found in the deeper urothelial layer and dermatan sulfate was predominantly found in deeper bladder layers. The role of GAGs present in the deeper layers remains unidentified. Consecutive immunofluorescence assays demonstrated that the GAG distribution on the urothelium was comparable between species (Janssen et al., 2013). Both urothelial cells and porcine bladder tissues closely mimic barrier components of the urothelium, thus urothelial dysfunction and injury observed in this thesis by three agents are clinically relevant to the patients and therefore supports the above toxicity mechanism.

The urothelium is one of the slowest growing epithelial cells in mammals under normal conditions, but when stimulated appropriately with growth signals especially during wound healing and carcinogenesis, the urothelium can undergo significant growth (Cohen and Ellwein, 1993; de Boer et al., 1994; Romih et al., 2001; Varley and Southgate, 2008; Walker, 1960). Due to its natural presence in the urine, epidermal growth factor (EGF) has been suspected to be one of the key *in vivo* growth signals for the urothelium (Fuse et al., 1991; Messing, 1992). Since epidermal growth factor receptors (EGFRs) are predominantly expressed by the basal cells of the urothelium, which is situated under the physical barrier imposed by the umbrella cells, the interaction between EGF ligand and its urothelial receptors is normally prevented (Messing, 1990; Messing et al., 1987; Romih et al., 2001).

Urothelial damage, resulting in loss of cells following treatment with all of the intravesical agents tested has been reported in this thesis. This can expose the basal cell layer, leading to urothelial proliferation and also hyperplasia which are demonstrated in rats treated with cyclophosphamide (CYP) injections (Romih et al., 2001; Zupancic et al., 2009) and in rats treated intravesically with the alkylating agents methyl methanesulphonate (MMS) and ethyl methanesulphonate (EMS) (Tudor et al., 1983). In a report by Romih et al. (2001), hyperplastic urothelium due to CYP-induced cell damage was fully restored to a normal three-layered urothelium two weeks after treatment. Therefore, it can be expected that the damaged urothelium observed in this thesis may potentially present with hyperplasia within this recovery period and subsequently produce higher levels of the stimulatory mediators ATP, ACh and PGE₂, which can cause further urothelial dysfunctions, abnormalities in detrusor activity and afferent nerve pathway. However, this was not the case in urothelial cell studies where the overall mediator levels were mostly unchanged one week following initial and repeat treatments. Therefore, it would be interesting to determine if the urothelium becomes hyperplastic *in vivo*, and if this increases the levels of urothelial mediators during a period recovery and also after a series of repeat intravesical treatments.

Anti-cholinergic medications such as oxybutynin are commonly used for the treatment of OAB. A recent study by Johnson et al. (2013) reported use of oxybutynin for symptom prophylaxis in bladder cancer patient receiving intravesical BCG in fact increased urinary frequency and burning on urination compared to

placebo. This highlights the need for an alternative approach to manage the adverse effects associated with intravesical treatment. Treating the inflammation and restoring the urothelial abnormalities may be a potential management of adverse effects reported by patients while maintaining efficacy of the intravesical chemotherapeutic agents that are currently available. Restoring or improving the protective bladder barrier has been utilised as a treatment approach for inflammatory bladder conditions (Bassi et al., 2011), including oral pentosan polysulfate and bladder instillations with hyaluronic acid, chondroitin sulfate and heparin. These treatments claim to repair the bladder GAG layer and some were recently shown to be efficacious in clinical studies (Damiano et al., 2011; Nickel et al., 2010; Nordling and van Ophoven, 2008; Parsons et al., 1993). However, restoring the barrier components or treating inflammation at early stages should be cautious and must be evaluated thoroughly before a trial as it may prevent uptake of these agents by tumour cells or halt the anti-tumour actions of these agents that appear to be effective at least in part by provoking an immune response.

Inflammation and abnormal detrusor activity

Inflammation of the urothelium may also suggest potential presentation of inflammation in the deeper detrusor muscle layer in which enhanced release of cytokines from the urothelium can also elicit inflammatory responses in detrusor muscle or it may be a concurrent effect. Previously, detrusor myocytes have not received significant attention in inflammatory diseases such as IC/PBS. However, many clinical studies using bladder biopsies from IC/PBS patients and animal models of bladder inflammation including CYP-induced cystitis found inflammatory changes in the bladder muscular layer, especially mast cell infiltration (Chuang et al., 2009; Girard et al., 2011; Sant et al., 2007). A recent study by Zhao et al. (2015) has demonstrated numerous mast cell infiltration in the detrusor muscle layer in a rat model of CYP-induced cystitis, indicating inflammation in this layer. In normal conditions, mast cells are rarely found in bladder muscle tissue and do not circulate in their mature form (Galli et al., 2005). However, when the detrusor myocytes are stimulated with IL-1 β or TNF- α , the detrusor myocytes can release inflammatory mediators, including IL-6, IL-8, monocyte chemoattractant protein-1 (MCP-1), and chemokine (C-C motif) ligand 5 (CCL5), which promote mast cell recruitment,

proliferation and maturation in the detrusor muscle layer (Bouchelouche et al., 2006a; Bouchelouche et al., 2006b; Galli et al., 2005).

Gap junctions are clusters of intercellular channels that provide direct cell-cell exchange of ions and small molecules, thus they have fundamental roles in excitable tissues (nerve and muscle) by allowing electrical transmission between cells (Connors and Long, 2004; Severs et al., 2004). Gap junction channels are composed of integral membrane proteins called connexins. In humans, there are 20 members of connexin gene family, of which the best-studied member is connexin 43 (Cx43) (Sohl and Willecke, 2003). The role of gap junctions for the function of bladder detrusor muscle is still a matter for debate but previously study has suggested a potential role in modulation of bladder voiding. Mori et al. (2005) reported a decrease in the number of gap junctions as well as a decrease in cellular membrane expression of Cx43 in the rat detrusor muscle with partial bladder outlet obstruction (P-BOO) as compared to control. Most importantly, in rats that demonstrated significantly low number of the gap junctions, no contraction was observed, suggesting that the normal signals that contribute to voiding function could be transported directly through the gap junction. Previous studies have demonstrated that the gap junctions in the urothelium or detrusor muscle can function as key modulators for non-neurogenic mechanisms of various hyperactive bladder conditions (Imamura et al., 2009; Imamura et al., 2013; Negoro et al., 2012; Negoro et al., 2013). Mice with CYP-induced cystitis have shown significantly upregulated expression of Cx43, more pronounced spontaneous contraction and frequent voiding compared to control. These overactive conditions were attenuated by gap junction inhibitors (Okinami et al., 2014). The effect of the three cytotoxic agents on the detrusor gap junction communication is unknown, but downregulation of gap junction proteins by doxorubicin has been reported in hearts (Zhang et al., 2011) and liver epithelial cells (Abdelmohsen et al., 2005). Modulation of connexin expression and gap junction intercellular communication (GJIC) by inflammatory cytokines has been demonstrated in various tissues. TNF- α decreased both Cx43 expression and GJIC in human epidermal keratinocytes and in rat glioma cells. Reduced expression of Cx43 and GJIC by transforming growth factor- β 1 (TGF β 1) has been reported in rat hepatic stellate cells. A study by Rama et al. (2006) has demonstrated a significant upregulation of Cx43 expression and GJIC after TGF β 1 stimulation. In contrast, Neuhaus and his colleagues (2009) reported significantly reduced Cx43

expression and GJIC by TGF β 1 in cultured human detrusor smooth muscle cells (hBSMC). A recent study using hBSMC by Heinrich et al. (2011) has demonstrated upregulation of GJIC after IL-6 stimulation. IL-4 and TGF β 1 decreased both Cx43 expression and GJIC, and TNF- α increased Cx43 expression. Therefore, it can be postulated that the affected muscle responsiveness by the three agents in this thesis may be due to inflammation-associated changes in the detrusor gap junctions. Accordingly, it would be advantageous to determine if there are changes in these gap junctions after treatment with the three agents of interest. This would confirm the potential role of the gap junctions as non-neurogenic mechanisms of OAB conditions following cytotoxic drug treatments.

Inflammation and sensitisation of afferent nerves / neuroplasticity

As demonstrated in tissues and cultured cells, urothelial cells are capable of secreting mediators including ATP, ACh and PGE₂ that can influence the underlying smooth muscle and nerves. These urothelial mediators are closely associated with overactivity and pain of the bladder, symptoms that patients report after intravesical treatment. Mediator release was shown to be affected by the three agents in this thesis but changes mostly returned to normal levels during recovery studies. In the setting of urothelial hyperplasia that may be induced by inflammation, as discussed above these mediators could potentially be further enhanced in clinical situations. However, in long-term prospective when inflammation and hyperplasia subside in patients there may be another mechanism that can contribute to the adverse effects.

Nerve growth factor (NGF) is a signaling protein expressed widely in various cells including urothelial cells, smooth muscle cells and mast cells (Steers et al., 1991). NGF affects bladder afferent nerves and is responsible for the growth and maintenance of sensory neurons (Steers, 2002). Previous studies have demonstrated a potential role of NGF as a chemical mediator of pathology-induced changes in C-fiber afferent nerve excitability and reflex bladder activity (Vizzard, 2000; Yoshimura, 1999). After spinal cord injury, increased level of NGF in the bladder (Vizzard, 2000, 2006) as well as increased levels in the lumbosacral spinal cord and dorsal root ganglia of rats have been reported (Satoshi et al., 2003). Also, various studies have demonstrated that administration of NGF into the spinal cord or

intravesical administration of NGF induce nociceptive response and bladder hyperactivity, and increases the firing frequency of dissociated bladder afferent neuron (Chuang et al., 2001; Dmitrieva et al., 1997; Lamb et al., 2004; Satoshi et al., 2003; Vizzard, 2006; Yoshimura et al., 2006; Zvara and Vizzard, 2007). Thus, NGF may lead to sensitisation of afferent nerves, enhancement of synaptic transmission, and pain sensation as well as increased urinary frequency (Figure 7.1).

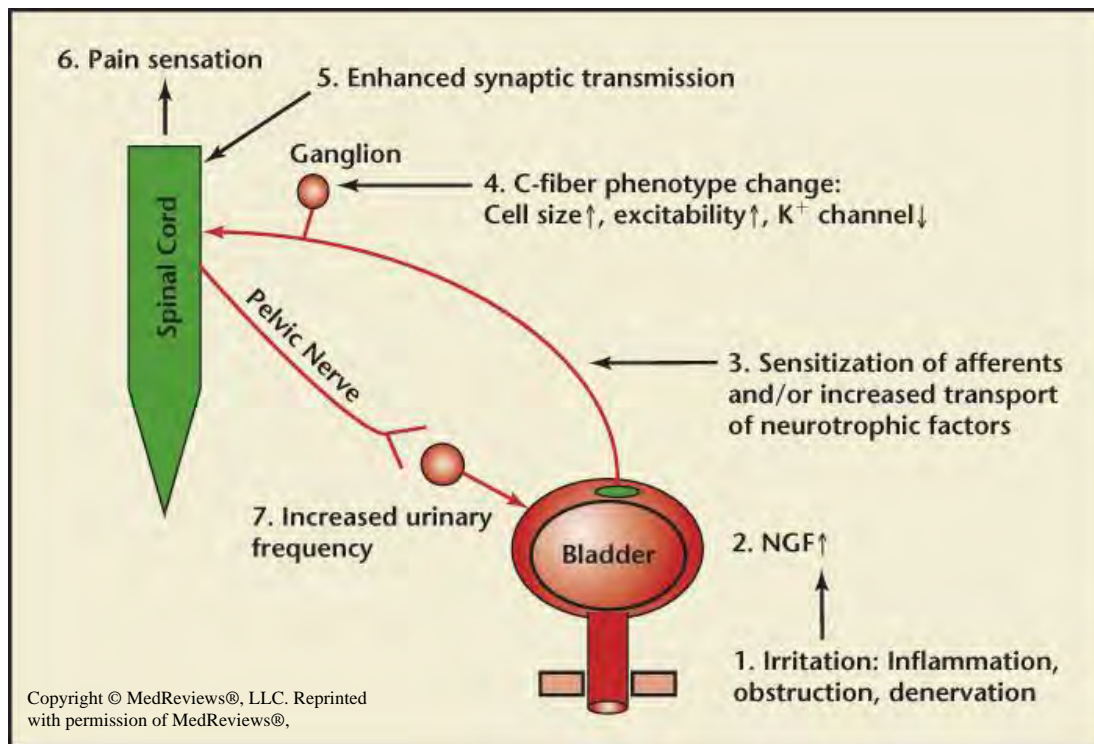


Figure 7.1: Nerve growth factor (NGF) is released from target cells under irritation due to inflammation, obstruction, or denervation. NGF sensitizes afferent nerves, enhances synaptic transmission, and produces pain sensation as well as increased urinary frequency (Kuo et al., 2010).

There is abundant evidence supporting that NGF may play a significant role in the link between inflammation and altered pain signaling. An increased expression of NGF has been reported in bladder biopsies from women with IC/PBS (Liu and Kuo, 2007; Lowe et al., 1997). A study by Lowe et al. (1997) has shown higher levels of NGF in samples obtained from patients with IC/PBS compared to control. Liu & Kuo (2007) has demonstrated significantly increased level of the NGF mRNA compared to control. In addition, Okragly et al. (1999) reported increased levels of NGF in the bladder tissue and urine of patients with IC/PBS. Furthermore, studies using inflammatory models of cystitis induced by CYP or LPS in rats or mice have

demonstrated increased levels of NGF production (Coelho et al., 2015; Gonzalez et al., 2005; Ho et al., 2011). A study by Dupont et al. (2001), which evaluated chemical (formalin), immune (LPS) and mechanical (chromic catgut) inflammation at various times compared to control bladders, has found that bladder inflammation in general resulted in a 50% increase in the levels of NGF production and 52% to 58% enlargement of peripheral neurons. Steers et al. (1991) also reported a potential correlation between NGF and neuroplasticity by an increase in both the dimensions of afferent and efferent neurons as well as increase in the levels of NGF in rats with bladder outlet obstruction compared to normal bladders. The findings of this thesis have identified signs of efferent nerve damage by decreased neurogenic responses in detrusor muscle tissues. This leaves the possibility of damaged nerves to proliferate and lead to hypertrophy, causing sensitisation of the efferent neurotransmission in the long-term. Therefore, further investigation into the effects of DOX, MMC and EPR on NGF production and morphology of sensory and motor neurons (neuroplasticity) may explain long-term symptoms including pain, frequency, and urgency that the patients experience even after the initial inflammatory stimulus has subsided or the resolution of bladder inflammation.

Concluding remarks

The work of this thesis has provided support for the previous findings that urinary bladder function changes in an age-associated manner. To the best of my knowledge, the results presented here is the first to report the effect of ageing in porcine bladder function. Consequently, this lead to the finding that aged porcine bladders, as compared to young bladders, is a better research model for mimicking bladder conditions of seniors that are more affected by bladder cancer. In this thesis, treatment with the intravesical chemotherapeutic agents, doxorubicin, MMC, and epirubicin, demonstrated various changes in local bladder mechanisms including alterations in the release of non-neuronal mediators from the urothelium, detrusor muscle contractility and relaxation, efferent neurotransmission, urothelial integrity, and inflammatory responses. One of the most important findings in this study was the persistent induction of inflammatory cytokines and it is my hypothesis that this may be the key to pathogenesis of the urological adverse effects reported in bladder cancer patients following intravesical treatment. Future research into the role of

inflammation could further our understanding of bladder toxicity mechanisms for these agents as well as other overactive and inflammatory bladder conditions.

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